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Metabolomics for Biomonitoring: Evaluation, Recommendations, and Test of Efficacy

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Abstract

Effective biomonitoring of aquatic environments is an integral component of ecosystem protection. The metabolome is an emerging indicator that may have the potential to serve as an ideal bioindicator due to its intrinsic capacity to detect physiological changes in organisms that could foreshadow ecosystem change. My thesis consisted of two complementary objectives. First, the metabolome was evaluated against several established criteria for an ideal bioindicator and found to have significant potential. Recommendations were also made regarding implementation of the metabolome into existing biomonitoring programs. Second, the metabolome of *Hexagenia spp.* was evaluated as a potential indicator for environmental effects of naphthenic acid exposure, a primary toxic component of oil sands process waters in the Athabasca oil sands region. Although further research is needed, the metabolome of *Hexagenia spp.* shows potential as an early warning indicator of naphthenic acid stress prior to the onset of ecologically significant change.

Keywords

Biomonitoring, *Hexagenia spp.*, Metabolomics, Naphthenic Acids, Oil Sands Process Waters, Toxicology

Co-Authorship Statement

This thesis contains content that will become two manuscripts. Sarah M. McKenzie will be the first author of both manuscripts due to undertaking the primary role in conducted research. Dr. Robert B. Brua will be the second author due to his co-supervision of both manuscripts and pivotal role in conducted research, in addition to providing funding. Natalie Izral will be included as the third author on the manuscript resulting from Chapter 2, due to her contributions to the conceptualization of the paper and contributions to literature review. Dr. Adam G. Yates will be the last author on both manuscripts due to his role as principal advisor and provision of the majority of funding.

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Chapter 1

1 General Introduction

1.1 Biomonitoring Approaches in the 21st Century

The influence of anthropogenic activities on the condition of aquatic environments has been well documented (Allan, 2004). For example, excess nutrient additions to agricultural lands result in eutrophication of aquatic ecosystems (Smith et al., 1999). Increased proportions of impervious surfaces have changed the hydrologic cycle in urban areas, as well as increasing pollutants entering streams (Arnold & Gibbons, 1996). Moreover, release of municipal wastewater introduces a myriad of synthetic compounds, such as endocrine disruptors and antibiotics to the environment, the precise effects of which are largely unknown (Caliman Florentina & Gavrilescu, 2009). Although it is widely recognized that natural aquatic ecosystems are at risk, the direction and magnitude of these changes are often unknown. Management agencies thus require tools that can effectively identify and monitor environmental conditions in order to protect aquatic environments and the ecosystems services they provide.

Biomonitoring, defined as the use of biological responses to identify and monitor changes in the environment (Reece & Richardson, 1999), is a method that has been used to quantify and characterize the magnitude and identity of anthropogenic impacts to stream ecosystems. Biomonitoring tools traditionally fall under two approaches (Munkittrick & McCarty, 1995). The first approach is toxicological in nature and examines relationships between stressors (e.g. metals) and specific biological responses at lower levels of biological organization, such as molecular cell responses (e.g. biomarkers). At lower levels of biological organization stress responses occur quickly and can therefore provide early warning signals of adverse effects (Kingett, 1985; Mitin, 1985; Graney & Giesy, 1986 Johnson et al., 1993). In contrast, the second approach assesses changes at higher levels of biological organization (i.e. population and community levels) that are then related to a potential underlying cause, such as sewage effluent. Changes at the community and population levels are considered to be more ecologically relevant than lower levels of

biological organization (Adams & Greeley, 2000). Both approaches have intrinsic strengths that are paramount to success in monitoring, however, these approaches are often poorly integrated, leaving gaps in knowledge and reducing capability for predictive management (Allan, 2007).

While community and population based approaches presents ecologically relevant data, they lacks the ability to warn of impending change and therefore presents the problem that detected change has the potential to be irreversible. Conversely, while approaches at lower levels of biological organization present timely data, indicators often are the result of contaminant exposure, and do not necessarily have direct causal linkages to changes at higher levels of the biological hierarchy. Although the biochemical level acts as an early indicator of aquatic stressors, it is difficult to extrapolate biochemical responses to higher levels of biological organization. Stress results in alteration of biochemical processes, but may not result in whole organism change. Therefore, only ecologically relevant changes (i.e. reproduction or growth) are reflected at population or community levels (Cairns et al., 1993; Munkittrick & McCarty, 1995). On the other hand, there can be uncertainty in linking cause and effect at the higher levels of biological organization. Changes at the community or population level may not have direct and observable links to causative agents or the underlying aquatic stressor (Munkittrick & McCarty, 1995). Linking biochemical changes in an organism to predictions of fitness and associated population responses introduces the possibility of predicting large-scale ecological changes before they occur within the natural environment (Underwood & Peterson, 1988). It is therefore necessary to integrate both higher level and lower level approaches in order to generate timely and ecologically relevant biomonitoring data that can be used to proactively manage the condition of our aquatic ecosystems.

1.2 Environmental Metabolomics

Environmental metabolomics is a rapidly emerging field that aims to relate changes in an organism's metabolome (i.e., the composition and relative quantity of various metabolites) to changes in its surrounding environment (Bundy et al., 2008; Lin et al., 2006; Viant, 2007).

Metabolomics has been successfully incorporated into toxicology frameworks and used to detect changes in the metabolome of target organisms (Lin et al., 2006; Viant et al., 2003; Wu & Wang, 2010). Metabolites are produced and consumed throughout thousands of individual biological processes, and therefore a shift in resource allocation will cause a change in the expression of these processes, resulting in a change in metabolite concentrations. Therefore, changes in the resource allocation in response to environmental conditions (and therefore stress) can be detected by analyzing the resulting metabolome.

Within metabolomics, both targeted and non-targeted analyses are prevalent. Targeted analyses specifically analyze a suite of metabolites of interest known to respond to stress variables. In contrast, non-targeted approaches use a more holistic method such that the metabolome is analyzed as a whole to determine significant changes in the overall status. Despite variations in data processing, the metabolome is acquired through one of two main methods; mass spectrometry (MS) or nuclear magnetic resonance (NMR) spectroscopy based sample analysis. Although MS methods offer greater sensitivity of low concentration metabolites, these methods are restricted to the analysis of metabolites fitting criterion, such as pKa and hydrophobicity requirements, and therefore NMR is a more common choice for broad holistic analysis (Pan & Raftery, 2007).

The metabolome has the benefit of being capable of establishing the linkages between different levels of the biological hierarchy through knowledge of known relationships between the metabolome and fitness. Relating sublethal responses (i.e. the metabolome) to fitness metrics ensures that the bioindicators in question are relevant from an environmental monitoring perspective. Fitness is defined as the ability to survive and reproduce, and therefore has intrinsic implications for population dynamics and species survival. Changes in specific metabolites can be related to biological processes involved in organism fitness, such as reproduction and growth, and therefore a decrease in metabolites associated with reproductive endpoints would suggest an overall decrease in organism fitness. Using existing knowledge on the often evolutionarily conserved physiology of organisms allows the generation of population level predictions from metabolomic data that can be validated with empirical evidence. Once mechanistic linkages are established within an organism or ecosystem, the metabolome could be applied as an early warning indicator, where changes in the metabolome are representative of organism stress and predictive of future population level responses.

Although use of the metabolome in biomonitoring is not yet widespread, the metabolome has been shown to be capable of differentiating between ecosystems exposed to differing levels of human activities. For example, Skelton et al. (2014) elucidated metabolomic changes distinctive of different effluent sources using caged fathead minnows (*Pimephales promelas*). Likewise, Davis et al. (2013) employed the metabolome of fathead minnows in their analysis on the effects of pulp and paper mill (PPM) effluent in the Great Lakes region, finding significant differences between exposed and control populations when effluent was being discharged. Additionally, in a marine environment, Cappello et al. (2013) detected clear alterations of the metabolome of mussels exposed to environments known to be anthropogenically polluted by different suites of contaminants. Davis et al. (2016) took an alternate approach, where the metabolome of fathead minnows was utilized and compared with chemical analyses of potential contaminants and used to prioritize potential chemicals of concern within the Great Lakes basin. Overall, the metabolome has seen successfully applied to elucidating environmental impacts, however, to our knowledge, a thorough evaluation of the metabolome for use in biomonitoring has not been conducted to date.

In order to operationalize the metabolome as a bioindicator it must be tested against a wide variety of stressors. While commonly used to detect the effects of metals in toxicological investigations (e.g., (Spann et al., 2011; Wu et al., 2011; Wu & Wang, 2010)), there are several current contaminants of concern in the environment that the metabolome has not been evaluated against. One current concern is that of the Oil Sands region in Western Canada, where extensive oil extraction and refinement operations are conducted. Several contaminants of concern are present in this area, including polyaromatic hydrocarbons (PAHs), heavy metals, and naphthenic acids (NAs). In particular, there is currently no specific indicator of sublethal NA exposure.

1.3 Naphthenic Acids and the Environment

The Athabasca Oil Sands region in western Canada contains one of the largest oil sands extraction operations in the world and is a major producer of oil and subsequently oil sands process waters (OSPWs). OSPWs are wastes generated by processing of extracted bitumen (a mixture of

hydrocarbons extracted from the ground), and generally amass a volume up to four times greater than that of subsequent refined oil products (Kannel & Gan, 2012). The Alberta government currently has in effect a policy prohibiting release of OSPW to aquatic environments (Giesy et al., 2010). One implication of this policy is the amassment of large volumes of OSPW with no definitive plan for disposal or remediation (van den Heuvel, 2015). OSPW are known to contain three major toxic components; heavy metals, polyaromatic hydrocarbons (PAHs), and naphthenic acids (McNeill et al., 2012; Pramanik, 2016), with naphthenic acids (NA) identified as the primary toxic component of OSPW (Verbeek et al., 1993). Naphthenic acids are a natural component of bitumen, however, NAs become concentrated in OSPWs through the bitumen extraction process (Headley & McMartin, 2004). Additionally, the formation of metal naphthenates, such as sodium naphthenate, is favourable in alkali environments (pKa ≈ 5) (A.P.I., 2012). Industry data suggests that sodium naphthenates (the sodium salts of naphthenic acids) comprise 69 ± 27% of naphthenic acids in tailings ponds, likely due to the introduction of sodium hydroxide during the extraction process (A. Alexander-Trusiak, pers. comm., 2017). Due to the large accumulation of OSPW and the need for a rapid solution to this issue, one proposed plan for OSPW disposal is the release of OSPW into adjacent waterways. Thus, there is an urgent need for information regarding aquatic toxicity of naphthenic acids and the development of bioindicators of exposure.

Naphthenic acids are classically defined as a family of saturated hydrocarbons containing a carboxylic acid functional group and one or more ring structures (typically separated by at least one carbon atom) (Headley et al., 2009; Headley et al., 2007; Hsu et al., 2000). The chemical formula for a classical naphthenic acid species is represented as $C_nH_{2n+z}O_2$, with n representing the number of carbon atoms present and z representing the number of hydrogen atoms lost due to unsaturation (assumed to be due to ring formation in classical NA, but could also be due to presence of pi bound carbon atoms). In this classical formula, n can be any positive, even integer, and z can be either zero or any even, negative integer. Classical naphthenic acids, also known as "O_2 naphthenic acids", have been found to comprise 73.6-82.6% of industrial naphthenic acid samples (Marentette et al., 2015). The remaining portion generally contain additional oxygen, nitrogen, and/or sulfur containing functional groups, as well as components of aromaticity or bridging cyclic structures (Grewer et al., 2010; Jones et al., 2012).

Naphthenic acid samples are known to be extremely heterogeneous in composition. Although generally under 500 Da regardless of source, differences in carbon number and Z family distribution are often apparent between samples, even when characterized from relatively similar sources (Grewer et al., 2010). Naphthenic acids are known to have varying toxicities, with lower molecular weight moieties exhibiting lower toxicities than their larger counterparts (Frank et al., 2008); potentially due to increased carboxylic acid content and therefore decreased hydrophobicity of the larger molecular weight species (Frank et al., 2009a). In contrast, Jones et al. (2011) identified toxicity gradients among different individual classes of NAs showing an increase in toxicity with increasing molecular weight, despite the lack of additional carboxylic functional groups. Several studies have shown microbial degradation to influence the concentration and composition, and therefore the toxicity, of naphthenic acid samples, with selective degradation of lower molecular weight compounds (Holowenko et al., 2002). Due to the differences in toxicity between naphthenic acid mixtures of varying compositions, the use of commercially derived NAs as OSPW surrogates for toxicity analysis has been criticized in the literature (West et al., 2011). Commercially derived samples have been shown to contain much higher portions of acyclic and single ringed (z=0,-2) NAs, while NAs derived from industry samples typically contain higher portions of z=-4 to -12 families (Marentette et al., 2015). Naphthenic acid samples have also been shown to contain various alkyl phenols in trace amounts, which are known estrogen receptor agonists (West et al., 2011). Due to the vast differences between industrial and commercially prepared NA samples, characterization of samples used for research purposes is paramount.

Aquatic environments are often at high risk of developing high pollutant concentrations due to factors such as collection and concentration of rainwater runoff. When considering the potential threat of naphthenic acids, aquatic environments surrounding oil sands development are of particular concern, due to their geographical proximity to oil sands operations. Concentrations of naphthenic acids in OSPW are reported upwards of 120 mg/L (Holowenko et al., 2002). In natural areas, NAs have been reported surrounding the oil sands in concentrations up to 10 mg/L. However, recent characterization of a number of sites surrounding oil sands operations by Ross et al. (2012) argue that concentrations are actually much lower, ranging from less than 2 µg/L in the Athabasca River to 173 µg/L in sediment pore water. Ross et al. (2012) explain that the variations in reported concentrations are potentially due to previous studies misclassifying other organic acids as NAs, and therefore concentrations of naphthenic acids in environmental samples may

actually be much lower than previously reported (Ross et al., 2012). In addition to being prevalent in areas where oil refining is occurring, NAs are also used for a variety of industrial applications, including, but not limited to, use as additives in agricultural insecticides, oil based paints, lubricating oils, and wood preservatives (Headley & McMartin, 2004). The potential prevalence of naphthenic acids in the environment is therefore not limited to areas where oil refinement is occurring, and actually represents a potentially widespread topic of environmental uncertainty. This further emphasizes the need for ecological knowledge surrounding the effects of NAs, especially in the case of a potential release of OSPW.

Some research has been conducted on acute toxicity of NAs, however knowledge is still limited. Naphthenic acid toxicity to four species, fathead minnow (*Pimephales promelas*), Cladoceran (*Daphnia magna*), algae (*Pseudokirchneriella subcapitata*), and bacteria (*Vibrio fischeri*), was recently investigated by Swigert et al. (2015). They found that fathead minnows were the most sensitive with a 96h LC50 of 5.6 mg/L. A similar comparative analysis of NA toxicity by Kinley et al. (2016) suggested a 7 day LC50 of 1.9 mg/L for P. promelas, with invertebrate (*Hyalella azteca, Ceriodaphnia dubia, Chironimus dilutes*) and macrophyte (*Typha latifolia*) species being less sensitive (7 day LC50 values ranging from 2.8 – 56.2 mg/L depending on species). In an assessment sodium naphthenate toxicity, Dokholyan and Magomedov (1983) exposed five Caspian fish species and several invertebrate species, including mollusks and worms, to sodium naphthenate. Based on their findings they concluded that environmental regulations be set at 0.15 mg/L in aquatic environments to prevent mortality in invertebrate species and at 0.8 mg/L when considering only fish species (Dokholyan and Magomedov 1983). A comparison of collected toxicity thresholds of a number of compounds similar in structure to naphthenic acids by Frank et al. (2009b) suggest a range of toxicity thresholds from 0.51 to 27.30 mM in D. magna, which was successfully predicted through the use of QSAR (quantitative structure activity relationships). Although acute toxicity information is useful, it does not provide the necessary context and ecological relevance that sublethal endpoints would address.

There is minimal knowledge regarding how naphthenic acids affect aquatic species at a sublethal level. NAs were determined to cause deformities in fertilized eggs in *P. promelas* and lower reproductive rates (Kavanagh et al., 2012; Marentette et al., 2015); a finding supported in several other fish species (Marentette et al., 2017; Peters et al., 2007). Sublethal impacts reported

from studies examining NA exposure in aquatic species have varied with reports of endocrine disruption (Kavanagh et al., 2012; Scarlett et al., 2012; Wang et al., 2015), increased oxidative stress (Marentette et al., 2017), liver toxicity (Nero et al., 2006; Scarlett et al., 2012), and disruption of several energy pathways (Melvin et al., 2013). Due to their surfactant properties, NA toxicity is currently assumed to be due to disruption of cell membranes, resulting in cell narcosis (Frank et al., 2009b). However, Frank et al. (2009b) have also identified inconsistencies of this assumption based on research findings that NA toxicity effects on the bacterium *Vibrio fischeri* decreases with molecular size, a finding contrary to what would be expected if a narcotic mode of action was present (Frank et al., 2008). Based on the knowledge gap presented surrounding the potential sublethal effects of NA, it is imperative that research be conducted to elucidate these effects.

Metabolomics has shown efficacy in determination of sublethal contaminant exposure effects and may therefore be suitable for use as a biomonitoring tool in the context of assessing environmental effects of naphthenic acids. Due to the prevalence of *Hexagenia spp.* in ecosystems across North America, including the Athabasca oil sands region, this invertebrate could represent an ideal organism for monitoring effects of bitumen extractions (Clifford & Boerger, 1974; Miner, 2014). *Hexagenia spp.* have been shown to have a positive relationship between body size and fecundity, and therefore the assumption that growth as an indicator of fitness is a viable metric for population level predictions is applicable, suggesting that this may be an appropriate indicator species for metabolomic monitoring (Clifford & Boerger, 1974; Giberson & Rosenberg, 1994; Hunt, 1951). *Hexagenia spp.* is also often deemed an organism of interest in terms of elucidating possible community level responses due to its position near the base of the food web as a consumer of detritus. Therefore, the removal of this organism has potentially strong implications on a community level as they are often a significant food source for many other aquatic and non-aquatic species (Miner, 2014). More specifically in relation to its potential as an organism of interest for monitoring effects of naphthenic acid, mayflies are a burrowing species that consume detritus. Naphthenic acids have been previously shown to bind to organic matter in sediment, and therefore the interaction between mayflies and sediment organic content may play a significant factor in elucidating potentially toxic interactions. *Hexagenia spp.* also exhibit a fast growth rate and short maturation period, meaning that changes to growth and fecundity as a result of NA contamination would appear quickly within the population. Due to its ecological relevance and ideal life history

attributes, *Hexagenia spp.* could therefore be suitable organisms for the development of a metabolomic signature of naphthenic acid exposure for environmental monitoring purposes.

1.4 Goals/Objectives

The goals of my thesis are twofold:

1. Introduce, describe, and evaluate the potential of the metabolome as a biomonitoring tool
2. Evaluate the efficacy of the *Hexagenia spp.* metabolome as a bioindicator of naphthenic acid exposure

By completing these objectives, I will generate knowledge regarding the usefulness of the metabolome in biomonitoring, as well as its potential monitoring applications in the oil sands region. Chapters 2 and 3 each address one these two main goals by addressing a suite of smaller goals.

Chapter 2: Metabolomics for Biomonitoring: A complete description and evaluation with case study and recommendations for implementation

1. Introduce and describe environmental metabolomics from a biomonitoring perspective
2. Evaluate the use of the metabolome as a potentially “ideal” bioindicator
3. Make recommendations for implementation of the metabolome into current biomonitoring approaches

Chapter 3: Case study: The effects of sublethal naphthenic acid exposure on the metabolome of *Hexagenia spp.*

1. Examine the metabolome for changes in organisms exposed to a gradient of predicted sublethal naphthenic acid concentrations

2. Examine the relationship between the metabolome and organism fitness in *Hexagenia spp.* by using growth as a surrogate measure of fitness

Ultimately, the completion of this thesis will allow the evaluation of both the theoretical and practical aspects of the utilization of metabolomics as a biomonitoring tool within current biomonitoring frameworks.

1.5 References

A.P.I. (2012). *Naphthenic acids category analysis and hazard characterization*. (Consortium Registration # 1100997). The American Petroleum Institute Petroleum HPV Testing Group.

Allan, J. D. (2004). Landscapes and riverscapes: the influence of land use on stream ecosystems. *Annual Review of Ecolology, Evolution, and Systematics., 35*, 257-284.

Arnold, C. L., & Gibbons, C. J. (1996). Impervious surface coverage: The emergence of a key environmental indicator. *Journal of the American Planning Association, 62*(2), 243-258. doi:10.1080/01944369608975688

Bundy, J. G., Davey, M. P., & Viant, M. R. (2008). Environmental metabolomics: a critical review and future perspectives. *Metabolomics, 5*(1), 3. doi:10.1007/s11306-008-0152-0

Caliman Florentina, A., & Gavrilescu, M. (2009). Pharmaceuticals, personal care products and endocrine disrupting agents in the environment – A Review. *CLEAN – Soil, Air, Water, 37*(4-5), 277-303. doi:10.1002/clen.200900038

Cappello, T., Mauceri, A., Corsaro, C., Maisano, M., Parrino, V., Lo Paro, G., . . . Fasulo, S. (2013). Impact of environmental pollution on caged mussels Mytilus galloprovincialis using NMR-based metabolomics. *Marine Pollution Bulletin, 77*(1), 132-139. doi:https://doi.org/10.1016/j.marpolbul.2013.10.019

Clifford, H. F., & Boerger, H. (1974). Fecundity of mayflies (Ephemeroptera), with special reference to mayflies of a brown-water stream of Alberta, Canada.

The Canadian Entomologist, 106(10), 1111-1119. doi:10.4039/Ent1061111-10

Davis, J. M., Collette, T. W., Villeneuve, D. L., Cavallin, J. E., Teng, Q., Jensen, K. M., . . . Ekman, D. R. (2013). Field-based approach for assessing the impact of treated pulp and paper mill effluent on endogenous metabolites of fathead minnows (*Pimephales promelas*). *Environmental Science & Technology, 47*(18), 10628-10636. doi:10.1021/es401961j

Davis, J. M., Ekman, D. R., Teng, Q., Ankley, G. T., Berninger, J. P., Cavallin, J. E., . . . Collette, T. W. (2016). Linking field-based metabolomics and chemical analyses to prioritize contaminants of emerging concern in the Great Lakes basin. *Environmental Toxicology and Chemistry, 35*(10), 2493-2502. doi:10.1002/etc.3409

Dokholyan, B., & Magomedov, A. (1983). Effect of sodium naphthenate on survival and some physiological-biochemical parameters of some fishes. *Journal of Ichthyology, 23*(6).

Frank, R. A., Fischer, K., Kavanagh, R., Burnison, B. K., Arsenault, G., Headley, J. V., . . . Solomon, K. R. (2009a). Effect of carboxylic acid content on the acute toxicity of oil sands Naphthenic acids. *Environmental Science & Technology, 43*(2), 266-271. doi:10.1021/es8021057

Frank, R. A., Kavanagh, R., Kent Burnison, B., Arsenault, G., Headley, J. V., Peru, K. M., . . . Solomon, K. R. (2008). Toxicity assessment of collected fractions from an extracted naphthenic acid mixture. *Chemosphere, 72*(9), 1309-1314. doi:http://dx.doi.org/10.1016/j.chemosphere.2008.04.078

Frank, R. A., Sanderson, H., Kavanagh, R., Burnison, B. K., Headley, J. V., & Solomon, K. R. (2009b). Use of a (quantitative) structure–activity relationship [(Q)Sar] model to predict the toxicity of Naphthenic acids. *Journal of Toxicology and Environmental Health, Part A, 73*(4), 319-329. doi:10.1080/15287390903421235

Giberson, D. J., & Rosenberg, D. M. (1994). Life histories of burrowing mayflies (*Hexagenia limbata and H. rigida, Ephemeroptera: Ephemeridae*) in a northern Canadian reservoir. *Freshwater Biology, 32*(3), 501-518. doi:10.1111/j.1365-2427.1994.tb01143.x

Giesy, J. P., Anderson, J. C., & Wiseman, S. B. (2010). Alberta oil sands development. *Proceedings of the National Academy of Sciences of the United States of America, 107*(3), 951-952. doi:10.1073/pnas.0912880107

Grewer, D. M., Young, R. F., Whittal, R. M., & Fedorak, P. M. (2010). Naphthenic acids and other acid-extractables in water samples from Alberta: What is being measured? *Science of the*

Total Environment, *408*(23), 5997-6010. doi:http://dx.doi.org/10.1016/j.scitotenv.2010.08.013

Headley, J. V., & McMartin, D. W. (2004). A review of the occurrence and fate of naphthenic acids in aquatic environments. *Journal of Environmental Science and Health, Part A, 39*(8), 1989-2010. doi:10.1081/ESE-120039370

Headley, J. V., Peru, K. M., & Barrow, M. P. (2009). Mass spectrometric characterization of naphthenic acids in environmental samples: A review. *Mass Spectrometry Reviews, 28*(1), 121-134. doi:10.1002/mas.20185

Headley, J. V., Peru, K. M., Barrow, M. P., & Derrick, P. J. (2007). Characterization of Naphthenic acids from Athabasca oil sands using electrospray ionization: The significant influence of solvents. *Analytical Chemistry, 79*(16), 6222-6229. doi:10.1021/ac070905w

Holowenko, F. M., MacKinnon, M. D., & Fedorak, P. M. (2002). Characterization of naphthenic acids in oil sands wastewaters by gas chromatography-mass spectrometry. *Water Research, 36*(11), 2843-2855. doi:http://dx.doi.org/10.1016/S0043-1354(01)00492-4

Hsu, C. S., Dechert, G., Robbins, W., & Fukuda, E. (2000). Naphthenic acids in crude oils characterized by mass spectrometry. *Energy & Fuels, 14*(1), 217-223.

Hunt, B. P. (1951). Reproduction of the Burrowing Mayfly, *Hexagenia limbata* (Serville), in Michigan. *The Florida Entomologist, 34*(2), 59-70. doi:10.2307/3492083

Jones, D., Scarlett, A. G., West, C. E., & Rowland, S. J. (2011). Toxicity of individual Naphthenic acids to *Vibrio fischeri*. *Environmental Science & Technology, 45*(22), 9776-9782. doi:10.1021/es201948j

Jones, D., West, C. E., Scarlett, A. G., Frank, R. A., & Rowland, S. J. (2012). Isolation and estimation of the 'aromatic' naphthenic acid content of an oil sands process-affected water extract. *Journal of Chromatography A, 1247*, 171-175. doi:http://dx.doi.org/10.1016/j.chroma.2012.05.073

Kannel, P. R., & Gan, T. Y. (2012). Naphthenic acids degradation and toxicity mitigation in tailings wastewater systems and aquatic environments: A review. *Journal of Environmental Science and Health Part A Toxic-Hazardous Substances & Environmental Engineering, 47*(1), 1-21. doi:10.1080/10934529.2012.629574

Kavanagh, R. J., Frank, R. A., Burnison, B. K., Young, R. F., Fedorak, P. M., Solomon, K. R., & Van Der Kraak, G. (2012). Fathead minnow (*Pimephales promelas*) reproduction is

impaired when exposed to a naphthenic acid extract. *Aquatic Toxicology, 116–117*, 34-42. doi:http://dx.doi.org/10.1016/j.aquatox.2012.03.002

Kinley, C. M., McQueen, A. D., & Rodgers Jr, J. H. (2016). Comparative responses of freshwater organisms to exposures of a commercial naphthenic acid. *Chemosphere, 153*, 170-178. doi:http://dx.doi.org/10.1016/j.chemosphere.2016.03.002

Lin, C. Y., Viant, M. R., & Tjeerdema, R. S. (2006). Metabolomics: Methodologies and applications in the environmental sciences. *Journal of Pesticide Science, 31*(3), 245-251. doi:10.1584/jpestics.31.245

Marentette, J. R., Frank, R. A., Bartlett, A. J., Gillis, P. L., Hewitt, L. M., Peru, K. M., . . . Parrott, J. L. (2015). Toxicity of naphthenic acid fraction components extracted from fresh and aged oil sands process-affected waters, and commercial naphthenic acid mixtures, to fathead minnow (Pimephales promelas) embryos. *Aquatic Toxicology, 164*, 108-117. doi:http://dx.doi.org/10.1016/j.aquatox.2015.04.024

Marentette, J. R., Sarty, K., Cowie, A. M., Frank, R. A., Hewitt, L. M., Parrott, J. L., & Martyniuk, C. J. (2017). Molecular responses of Walleye (Sander vitreus) embryos to naphthenic acid fraction components extracted from fresh oil sands process-affected water. *Aquatic Toxicology, 182*, 11-19. doi:http://dx.doi.org/10.1016/j.aquatox.2016.11.003

McNeill, S. A., Arens, C. J., Hogan, N. S., Köllner, B., & van den Heuvel, M. R. (2012). Immunological impacts of oil sands-affected waters on rainbow trout evaluated using an in situ exposure. *Ecotoxicology and Environmental Safety, 84*, 254-261. doi:http://dx.doi.org/10.1016/j.ecoenv.2012.07.016

Melvin, S. D., Lanctôt, C. M., Craig, P. M., Moon, T. W., Peru, K. M., Headley, J. V., & Trudeau, V. L. (2013). Effects of naphthenic acid exposure on development and liver metabolic processes in anuran tadpoles. *Environmental Pollution, 177*, 22-27. doi:http://dx.doi.org/10.1016/j.envpol.2013.02.003

Miner, A. (2014). *Hexagenia limbata*. *Animal Diversity Web.* Retrieved from http://animaldiversity.org/accounts/Hexagenia_limbata/

Nero, V., Farwell, A., Lee, L. E. J., Van Meer, T., MacKinnon, M. D., & Dixon, D. G. (2006). The effects of salinity on naphthenic acid toxicity to yellow perch: Gill and liver histopathology. *Ecotoxicology and Environmental Safety, 65*(2), 252-264. doi:http://dx.doi.org/10.1016/j.ecoenv.2005.07.009

Pan, Z., & Raftery, D. (2007). Comparing and combining NMR spectroscopy and mass spectrometry in metabolomics. *Analytical and Bioanalytical Chemistry, 387*(2), 525-527. doi:10.1007/s00216-006-0687-8

Peters, L. E., MacKinnon, M., Van Meer, T., van den Heuvel, M. R., & Dixon, D. G. (2007). Effects of oil sands process-affected waters and naphthenic acids on yellow perch (*Perca flavescens*) and Japanese medaka (*Orizias latipes*) embryonic development. *Chemosphere, 67*(11), 2177-2183. doi:http://dx.doi.org/10.1016/j.chemosphere.2006.12.034

Pramanik, S. (2016). Review of biological processes in oil sands: a feasible solution for tailings water treatment. *Environmental Reviews, 24*(3), 274-284. doi:10.1139/er-2015-0088

Reece, P. F., & Richardson, J. S. (1999). *Biomonitoring with the reference condition approach for the detection of aquatic ecosystems at risk.* Paper presented at the Proceedings of a Conference on the Biology and Management of species and habitats at risk.

Ross, M. S., Pereira, A. d. S., Fennell, J., Davies, M., Johnson, J., Sliva, L., & Martin, J. W. (2012). Quantitative and qualitative analysis of naphthenic acids in natural waters surrounding the Canadian oil sands industry. *Environmental Science & Technology, 46*(23), 12796-12805. doi:10.1021/es303432u

Scarlett, A. G., West, C. E., Jones, D., Galloway, T. S., & Rowland, S. J. (2012). Predicted toxicity of naphthenic acids present in oil sands process-affected waters to a range of environmental and human endpoints. *Science of the Total Environment, 425*, 119-127. doi:http://dx.doi.org/10.1016/j.scitotenv.2012.02.064

Skelton, D. M., Ekman, D. R., Martinović-Weigelt, D., Ankley, G. T., Villeneuve, D. L., Teng, Q., & Collette, T. W. (2014). Metabolomics for in situ environmental monitoring of surface waters impacted by contaminants from both point and nonpoint sources. *Environmental Science & Technology, 48*(4), 2395-2403. doi:10.1021/es404021f

Smith, V. H., Tilman, G. D., & Nekola, J. C. (1999). Eutrophication: impacts of excess nutrient inputs on freshwater, marine, and terrestrial ecosystems. *Environmental Pollution, 100*(1), 179-196. doi:https://doi.org/10.1016/S0269-7491(99)00091-3

Spann, N., Aldridge, D. C., Griffin, J. L., & Jones, O. A. H. (2011). Size-dependent effects of low level cadmium and zinc exposure on the metabolome of the Asian clam, Corbicula fluminea. *Aquatic Toxicology, 105*(3), 589-599. doi:https://doi.org/10.1016/j.aquatox.2011.08.010

Swigert, J. P., Lee, C., Wong, D. C. L., White, R., Scarlett, A. G., West, C. E., & Rowland, S. J. (2015). Aquatic hazard assessment of a commercial sample of naphthenic acids. *Chemosphere, 124*, 1-9. doi:http://dx.doi.org/10.1016/j.chemosphere.2014.10.052

Taylor, N., Gavin, A., & Viant, M. (2018). Metabolomics discovers early-response metabolic biomarkers that can predict chronic reproductive fitness in individual *Daphnia magna*. *Metabolites, 8*(3). doi:10.3390/metabo8030042

Underwood, A., & Peterson, C. (1988). Towards an ecological framework for investigating pollution. *Mar. Ecol. Prog. Ser., 46*(1), 227-234.

van den Heuvel, M. R. (2015). In Response: An academic perspective on the release of oil sands process–affected water. *Environmental Toxicology and Chemistry, 34*(12), 2682-2684. doi:10.1002/etc.3140

Verbeek, A., Mackay, W., & MacKinnon, M. (1993). *Isolation and characterization of the acutely toxic compounds in oil sands process water from Syncrude and Suncor*. Retrieved from

Viant, M. R. (2007). Metabolomics of aquatic organisms: the new 'omics' on the block. *Marine Ecology Progress Series, 332*, 301-306. Retrieved from http://www.int-res.com/abstracts/meps/v332/p301-306/

Viant, M. R., Rosenblum, E. S., & Tjeerdema, R. S. (2003). NMR-based metabolomics: A powerful approach for characterizing the effects of environmental stressors on organism health. *Environmental Science & Technology, 37*(21), 4982-4989. doi:10.1021/es034281x

Wang, J., Cao, X., Huang, Y., & Tang, X. (2015). Developmental toxicity and endocrine disruption of naphthenic acids on the early life stage of zebrafish (Danio rerio). *Journal of Applied Toxicology, 35*(12), 1493-1501. doi:10.1002/jat.3166

West, C. E., Jones, D., Scarlett, A. G., & Rowland, S. J. (2011). Compositional heterogeneity may limit the usefulness of some commercial naphthenic acids for toxicity assays. *Science of the Total Environment, 409*(19), 4125-4131. doi:http://dx.doi.org/10.1016/j.scitotenv.2011.05.061

Wu, H., Liu, X., Zhao, J., & Yu, J. (2011). NMR-based metabolomic investigations on the differential responses in adductor muscles from two pedigrees of Manila Clam *Ruditapes philippinarum* to cadmium and zinc. *Marine Drugs, 9*(9), 1566. Retrieved from http://www.mdpi.com/1660-3397/9/9/1566

Wu, H., & Wang, W.-X. (2010). NMR-based metabolomic studies on the toxicological effects of cadmium and copper on green mussels Perna viridis. *Aquatic Toxicology, 100*(4), 339-345. doi:http://dx.doi.org/10.1016/j.aquatox.2010.08.005

Chapter 2

2 Metabolomics for Biomonitoring: A complete description and evaluation with case study and recommendations for implementation

2.1 Introduction

A requirement of effective management, restoration, and protection of aquatic systems is the collection of ecological information through biomonitoring. Biomonitoring is defined as the use of biological responses to identify and monitor changes in the environment (Reece & Richardson, 1999). Tools for biomonitoring traditionally fall under one of two distinct approaches (Munkittrick & McCarty, 1995).

The first approach examines relationships between stressors and specific biological responses at lower levels of biological organization (e.g. cell or tissue levels). Responses to stress, including biochemical and physiological responses of individual organisms, occur quickly at lower levels of biological organization and therefore provide early warning of potential ecological effects (Adams et al., 1989). Although responses at lower levels of biological organization act as early indicators of aquatic stress, it is often difficult to forecast if changes will result in ecologically relevant effects at population or community levels (Cairns et al., 1993; Munkittrick & McCarty, 1995).

The second approach studies effects of environmental stressors on higher levels of biological organization (i.e. population and community levels). Changes at the population and community levels provide a holistic view of the ecosystem and are considered more ecologically relevant than lower levels of biological organization (Adams & Greeley, 2000). However, changes at the community or population level may not have direct and observable links to underlying stressors (Munkittrick & McCarty, 1995). Another criticism of community or population based assessments is that ecological changes must have already occurred in order to be detected in an environment, and have the potential to be complex, negative, and irreversible.

The individual limitations of current bioassessment approaches mean that neither approach can independently meet all biomonitoring objectives. For example, the population and community based approaches have successfully been applied for status and trends assessment driven monitoring, but have proven difficult to apply to causal effect assessments (Brua et al., 2015). Similarly, biochemical approaches are useful for identifying mechanistic linkages between stressors and biological responses, but are limited in their ability to predict changes in ecological status. A proposed solution is integrating these two approaches to address environmental effects of stressors, by simultaneously monitoring several levels of the biological hierarchy. However, there has been limited application of such a framework, resulting from the extensive logistical and financial resources that would be required to monitor the diverse suite of bioindicators needed to integrate the two approaches. There would thus be benefits to having a single bioindicator that inherently connects multiple levels of biological organization.

Metabolomics is a method that presents a unique opportunity to address gaps in biomonitoring approaches by holistically analyzing biochemical responses that are often precursors to population and community effects. As resource allocation shifts in an organism, these changes are reflected in the metabolome and have shown to be indicative of changes in observable organism fitness (Hines et al., 2010; Taylor et al., 2018). As a biomonitoring method, metabolomics thus has the potential to provide ecological information linking all levels of the biological hierarchy. Furthermore, information derived from metabolomics can be used in a similar manner as traditional community-based metrics, while also presenting opportunities for delineation of mechanistic links between environmental stressors and ecological change. However, the potential application of metabolomics as an effective biomonitoring tool has not been rigorously assessed and it is not well recognized by aquatic managers and practitioners as an alternative to current biomonitoring methods.

The goals of this paper are threefold. First, we introduce and describe the field of metabolomics and its associated conceptual underpinnings, as well as summarizing the key methodologies for generating metabolomic data. Second, we evaluate the potential of the metabolome to serve as an ideal bioindicator. Third, we make recommendations for implementation and integration of metabolomics within existing frameworks and present a case study illustrating the potential of the metabolome to assess ecological change.

2.2 Environmental Metabolomics

Environmental metabolomics relates environmental conditions to an organism's metabolome, which is the summation of all metabolites (i.e., small molecules related to biochemical processes) in a sample present at a given time (Bundy et al., 2008; Lankadurai et al., 2013; Viant, 2008). Assessment of the metabolome is undertaken through one of two approaches: targeted and non-targeted metabolomics. Non-targeted metabolomics uses the whole metabolome to identify differences between individuals or populations exposed to different environments in a qualitative manner. The metabolome can then be further analyzed to identify specific areas of the metabolome, and therefore specific metabolites, that are associated with environmental factors. Targeted metabolomics uses *a priori* selected metabolites within the metabolome to ascertain organism response to an environmental stressor (Gowda & Djukovic, 2014). Targeted metabolomics can either use a qualitative approach where changes are analyzed relative to control samples or concentrations of individual metabolites can be measured to provide a quantitative assessment. Although targeted and non-targeted methods are designed to answer different assessment questions, the approaches utilize similar sampling and analytical techniques.

Samples obtained for metabolomic analyses can range from fluid (e.g. urine, saliva) and tissue (e.g. liver, muscle) samples to one or more whole organisms. Collection of metabolomic samples varies widely, particularly in relation to organism size, as some samples may require organism dissection. Additionally, availability of sample will impact potential analysis techniques, as some techniques have specific mass requirements. Regardless of preliminary sample collection methods, samples should be flash frozen immediately to prevent any alterations to the metabolome (e.g. effects of handling stress on whole organisms, or effects of cell death in dissected tissue). Metabolite extractions from stored samples follow techniques that are simple and practical to execute (Beckonert et al., 2007; Lin et al., 2007; Wu et al., 2008) and once extracted, metabolites can be frozen and archived indefinitely before analysis.

Extracted metabolites are measured by mass spectrometry (MS) or nuclear magnetic resonance (NMR) spectroscopy. MS methods offer greater sensitivity of low concentration metabolites, and is thus most commonly used when metabolites of interest are known or predicted to be of low concentration. Moreover, in cases of large molecules, such as lipids, MS offers metabolite specificity and therefore higher probabilities of identifying individual metabolites.

However, MS methods are restricted to analysis of metabolites fitting specific criterion resulting in a narrower window of analyzed metabolites. This restriction potentially excludes some metabolites when using non-targeted approaches. In contrast, NMR analyses produce more holistic datasets and is more commonly used when a whole metabolome approach is required. In addition, small polar molecules (e.g. amino acids) are more readily identified by their NMR signature. However, low concentration metabolites are often masked by higher concentration metabolites and therefore not detected (Pan & Raftery, 2007). Compared to MS, NMR also has the benefit of being a non-destructive analysis technique, such that the sample can be used for multiple analyses and also archived for later assessment including MS analyses. Each analytical technique allows several options to optimize for specific project requirements (e.g. coupling to chromatographic separation techniques and use of specialized probes) to allow for further control over data produced.

NMR and MS methods produce a spectrum (Fig. 1) containing all of the information provided by the metabolome. Although the actual data produced differs between NMR and MS, both spectra are representative of an overlapping set of signals from all metabolites present in a sample. A MS spectrum is comprised of discrete peaks representing mass to charge ratios (m/z ratios) of ionized metabolites and spectral peak height is proportional to metabolite abundance at a specific m/z ratio. In contrast, a NMR spectrum comprises continuous data representing the relaxation frequencies (Hz) of individual protons, and peak areas are representative of metabolite abundance. In the case of NMR data, the spectrum is reduced into sample "bins" for data analysis. In both cases, data is analyzed by common statistical procedures, such as Principal Component Analysis or Partial Least Squares Discriminant Analysis for non-targeted analysis, whereas more specific peak finding programs (e.g., Chenomx (Chenomx NMR Suite v8.3 2017)) can be used for targeted analyses.

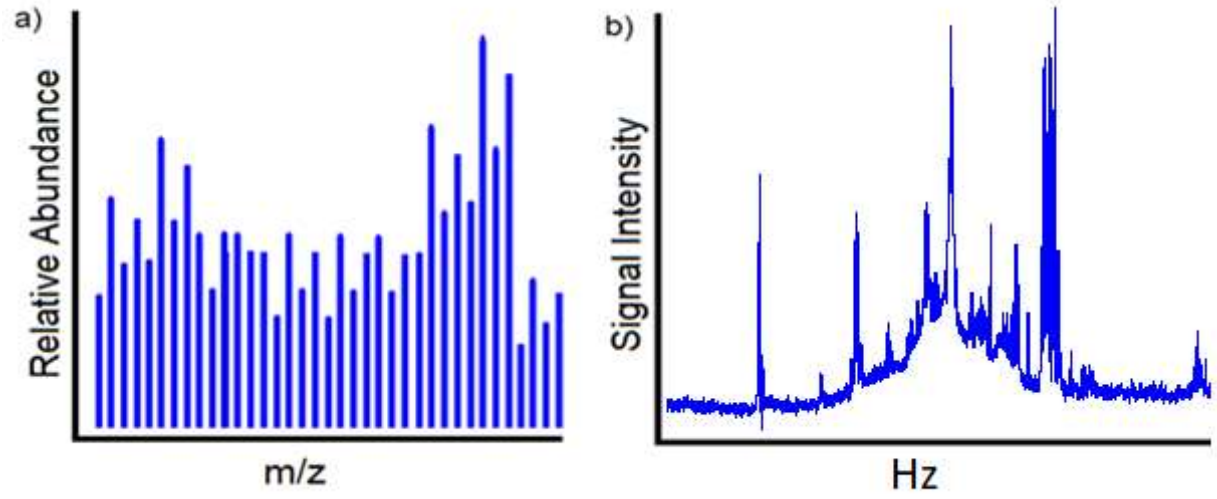

Figure 1: Metabolomic spectra produced by a) Mass Spectrometry and b) Nuclear Magnetic Resonance Spectroscopy methods

2.3 Evaluation of Metabolomics as a Tool for Biomonitoring

We evaluated the strengths and weakness of metabolomics as a biomonitoring tool by applying nine criteria, adapted from Bonada et al. (2006) (Table 1). Criteria were grouped into three categories: 1) Theoretical considerations; 2) Capabilities and reliability; and, 3) Practical considerations. For further rationalization of the criteria we direct the reader to Bonada et al. (2006). Our assessment of the established criteria was based on a review of existing metabolomic literature. We deemed the metabolome to meet the criteria when all reviewed literature offered support. If the literature reviewed was equivocal or no literature was available, the criteria was regarded as needing more research to reach a definitive conclusion. If no support was found in existing literature, the criteria was assessed as not met.

Based on our evaluation (see text below) of the metabolome as an indicator, we found that the metabolome meets six of the nine criteria and more research is needed for the four remaining criteria (Table 1). As such, we conclude that metabolomics is an effective method for use in biomonitoring.

Table 1: Evaluation of the metabolome as a biomonitoring tool as per criteria established by Bonada et al. (2006). MRN indicates that more research is needed to reach a definitive assessment.

Theoretical Considerations	**Assessment**
Derived from sound theoretical concepts in ecology	✓
A priori predictive	✓
Potential to assess ecological functions	✓
Capabilities and Reliability	
Capable of discriminating overall human impact (i.e., to identify anthropogenic disturbance) and discriminating different types of human impact (i.e., to identify specific types of anthropogenic disturbance)	✓
Reliable indication of changes in overall human impact and changes in different types of human impact	MRN
Human impact indication on linear scale	MRN
Large-scale applicability (across ecoregions or biogeographic provinces)	MRN
Practical Considerations	
Simple sampling protocol	✓
Low cost for sampling, sorting, and analysis	✓

2.3.1 Theoretical Considerations of Metabolomics in Biomonitoring

Support for the use of the metabolome as a bioindicator is grounded in classic life history theory, whereby organism fitness is impacted as it forfeits success in one area (e.g. reproduction) in favour of success in another area (e.g. growth) when resources are limited (Stearns, 1989). Shifts in resource allocation alter the expression of various metabolic pathways and the relative quantities of associated metabolites (Brown et al., 2004; Peñuelas & Sardans, 2009b), theoretically linking the metabolome to organism fitness. Moreover, the metabolome-fitness linkage has been demonstrated empirically. For example, Hines et al. (2010) linked metabolite changes in mussels to decreased organism fitness using scope for growth metrics. Likewise, Taylor et al. (2018) discovered metabolites predictive of declining reproductive fitness after chronic exposure to chemicals in *Daphnia magna.* Although well-grounded in sound theoretical concepts, more studies would assist to empirically confirm the universality of the linkage between fitness and the metabolome.

The metabolome is regarded as having potential for *a priori* predictive capacity of change at higher levels of biological organization (e.g. population), as individual biochemical adaptations are precursory to changes in organism physiology and population dynamics (Bahamonde et al., 2016; Lankadurai et al., 2013). Thus, as a bioindicator, the metabolome has the ability to identify potential effects of a stressor before those effects become evident in traditional fitness metrics (e.g. growth and fecundity)(Lankadurai et al., 2013). However, *a priori* predictions require identification of mechanistic linkages relating specific changes in the metabolome to fitness effects before the metabolome can be used for diagnostic assessments of environmental impacts. Taylor et al. (2018) provide an example of the development of predictive models using the metabolome. Thus, although the metabolome has clear theoretical potential to be an indicator with *a priori* predictive capacity, there is need for additional empirical evidence to determine the metabolic shifts that generate ecologically relevant changes.

The metabolome is indicative of the cumulative outcome of several biological functions (e.g. growth, reproduction). All of these biological functions are cumulatively indicative of the health and behavior of an individual organism (Peng et al., 2015). Moreover, if such metabolome changes are reflected at a population or community scale, the metabolome could manifest ecologically as a change in ecosystem function. For example, decomposition in small streams is

partly controlled by the activity rate of shredding invertebrates. A decline in the rate of shredding activity due to individual organism response to stress would thus be reflected in metabolomic changes. To our knowledge there has been no direct evaluation of these linkages, however the theoretical basis of these assumptions is evident. Therefore, while not a direct functional indicator, the metabolome is theoretically capable of assessing ecological function.

2.3.2 Capabilities and Reliability of Metabolomics in Biomonitoring

Numerous studies in metabolomics have successfully discriminated changes in overall human impact through comparisons of impacted and non-impacted sites (Gago-Tinoco et al., 2014; Ji et al., 2013; Li et al., 2014; Wagner et al., 2017; Wu et al., 2017). Caged mussels were found to undergo significant metabolic alterations when exposed to an environment contaminated with industrial petrochemicals (Cappello et al., 2013). Distinct metabolomic responses have also been found between similar stressors. For example, (Khan et al., 2016) found distinct metabolomes in *Carassius auratus gibelio* exposed to lead and cadmium, including an additive effect upon simultaneous exposure to both metals. Likewise, Skelton et al. (2014) detected differences in the metabolomes of *Pimephales promelas* exposed to point and non-point source contamination from different land use types. These findings indicate that the metabolome can be an effective bioindicator of overall human impacts and can distinguish between different types of human impacts.

To be a reliable indicator, changes in the metabolome associated with human impact must be distinguishable from inherent population variability. Additionally, it is necessary for the metabolome to exhibit a repeatable response to the same stressor. Several confounding factors (e.g. age, sex, tissue) can lessen the reliability of results. For example, Lin et al. (2009) found that effects of chemical dispersants on the metabolome of *Oncorhyncus tshawytscha* were masked by variation in age. Wu et al. (2017), also found that mussel response to cadmium was dependent on life cycle stage. Moreover, metabolomic analyses in mussels showed sex-specific differences in the basal metabolome and in their metabolomic response to contaminant exposures (Ji et al., 2016;

Ji et al., 2013). Sex effects in metabolomics have also been observed in sea snails and fish (Lu et al., 2017; Qiao et al., 2016). Differential responses with varying tissue type is also commonly observed in the literature as a potential confounding factor to the detection of environmental effects (Hu et al., 2015; Lin et al., 2009). For example, Li et al. (2014) found differences in the exposed metabolome of goldfish when heart, kidney, and brain tissues were analyzed, but not liver tissue, despite liver tissue being a common target organ for metabolomic investigations (Katsiadaki et al., 2010; Qiao et al., 2016). Seasonal variation in life history factors, such as reproductive cycles, should be also taken into consideration (Martyniuk, 2018). In addition to within population variation, several authors warn that between population variation in natural settings may be too large to permit identification of a consistent biological response to environmental change (Bahamonde et al., 2016; Bencic, 2015; Martyniuk, 2018; Miller, 2007). However, to our knowledge no widespread metabolomic studies have been conducted supporting such assertions. Moreover, Simmons Denina et al. (2015) propose several statistical methods to overcome the limitations associated with significant between population variability. Research describing the magnitude and drivers of within and between population variability in the metabolome is needed to allow design of effective sampling regimes for monitoring programs (Hines et al., 2007; Martyniuk, 2018; Morrison et al., 2007; Peñuelas & Sardans, 2009a; Viant, 2007).

A linear response in a bioindicator is advantageous because the indicator is capable of reflecting incremental change across the full range of stressor exposure. Few dose-response relationships have been derived for the metabolome of aquatic organisms. Santos et al. (2010) found a linear dose-dependent relationship between copper exposure and the whole metabolome in mature male stickleback (*Gasterosteus aculeatus*). Hu et al. (2015) found dose-dependent responses in female *Cyprinus flammans* to sublethal copper exposure, however results were not linear. In contrast, Kovacevic et al. (2016) exposed *Daphnia magna* to concentration gradients of three different stressors and did not observe dose-dependent metabolomic responses. It must also be noted that while linear responses are ideal and certainly show promise for development, not all stressors have dose-dependent toxic modes of action and therefore a response on a linear scale may not be achievable for all stressor types.

Bioindicators that are applicable at large spatial scales (e.g. ecoregions or biogeographic provinces) can allow data comparison and integration for regional to continental scale monitoring

programs. The metabolome is expected to be applicable across many spatial scales because metabolic pathways, unless altered by genetic adaptations to habitat difference, should be spatially conserved (Connon et al., 2012; Gago-Tinoco et al., 2014). However, our literature review did not uncover any studies that have tested this theoretical relationship. Further efforts should be aimed at analyzing the metabolome of organisms exposed to similar stressors across large spatial scales.

2.3.3 Practicality of Metabolomics in Biomonitoring

Collection of samples for metabolomic analyses can be undertaken using the same sampling methods as many biomonitoring tools (e.g. invertebrate communities, fish physiological metrics). Metabolomic sample collections do not require any specialized skills or equipment aside from a portable nitrogen dewar for immediate flash freezing of samples. An important consideration, however, is that some analytical methods have sample mass requirements that may dictate the type of analysis and instrumentation that can be used to generate metabolomic data. Methods for sample processing are easily accessible and simple to execute. Ultimately, however, the simplicity of metabolomics as a biomonitoring tool is comparable to current approaches.

Costs for metabolomic analyses range widely, as total costs depend on the extent of services required and the method used for analysis. Several commercial and academic laboratories offer routine metabolomic analyses, with services ranging from simple sample analysis to full project support throughout the planning stages through to data analysis. Costs for metabolomic services range from as low as $70 USD for a single untargeted sample analysis to several thousand dollars for full service metabolomics. Metabolomic analyses do require the use of expensive and specialized analysis equipment (upwards of $1M USD is required to purchase an NMR spectrometer or a mass spectrometer). If equipment was available for internal use sample analysis costs could be reduced dramatically to as little as $5 USD per sample. Most universities have the instrumentation required for analysis and, therefore, monitoring programs would benefit from integrated partnerships reducing costs. Additionally, government agencies and universities would have the resources to overcome initial costs and provide analysis as a service to monitoring programs. This is similar to biomonitoring tools currently in use (e.g. Canadian Aquatic

Biomonitoring Network, (Canada, 2012)). Overall, costs associated with metabolomics are comparable to those of current biomonitoring tools and would not represent a significant increase in costs.

2.4 Recommendations and Implementation

Our evaluation has demonstrated that the metabolome has many characteristics of an ideal biomonitoring tool. Moreover, in addition to meeting the identified criteria, the metabolome also has significant advantages over many current bioindicators including the ability to: 1) link several levels of the biological hierarchy; 2) be an early warning indicator, and; 3) eventually be used for causal diagnosis of stream impacts. Although more research is needed in order for the practical realization of its complete suite of benefits, it is currently operational for use in traditional status assessments.

Status assessments currently use one of several established approaches, whereby a test site is compared to one or more reference sites and deviation from reference condition is indicative of an anthropogenic impact. Some examples of the types of approaches used include Before/After/Control/Impact and gradient designs (Brua et al., 2015). The overarching theme of these kind of assessments is to identify a change from reference condition. An illustration of how the metabolome can be used to identify deviations from reference condition in the context of currently utilized frameworks is presented below (Textbox 1). In this study, we showed how the metabolome of Hydropsychidae larvae shifted upon exposure to municipal effluent prior to recovering to upstream reference conditions at a site more than 100 km downstream.

Text Box 1: Test of the metabolome as a bioindicator of municipal wastewater effluent exposure in the South Saskatchewan River

The South Saskatchewan River is an important water resource in the Canadian Prairies. The river receives treated municipal wastewater effluent from the City of Saskatoon (Pop'n ≈ 245 000; (Statistics Canada, 2017)) Wastewater Treatment Plant. The plant has class 4 certification (Saskatoon, 2018) and provides tertiary treatment, however, increases in nutrients and pharmaceuticals and personal care products (PPCPs) have been measured downstream of the plant outfall, which discharges more than 86,000 m^3 of effluent per day (Table 1). I assessed potential effects of the plant on ecological status by comparing the metabolome of Hydropsychidae larvae downstream of the plant to an upstream reference site.

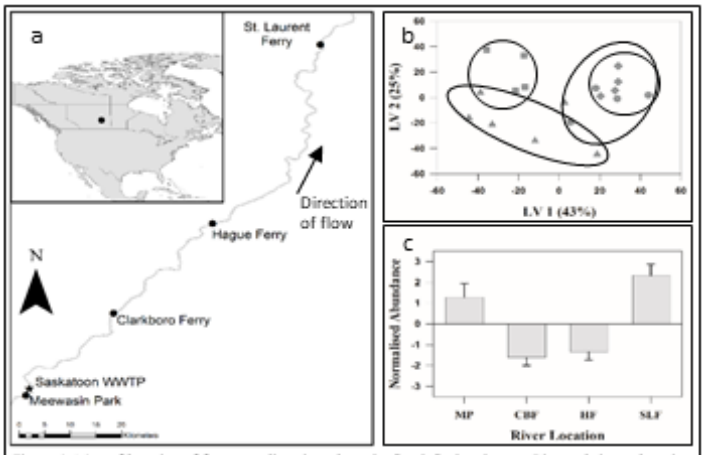

Figure 1: Map of location of four sampling sites along the South Saskatchewan River relative to location of Saskatoon Wastewater Treatment Plant (a). PLS-DA plot (b) showing predicted groupings (black circles) of Hydropsychidae metabolomes collected at the four sampling locations (Meewasin Park [•]; Clarksboro Ferry [■]; Hague Ferry [▲]; St. Laurent Ferry [♦]). Plot showing means and standard errors of normalized abundance of alanine in Hydropsychidae collected at four sampling sites.

Hydropsychidae larvae were collected by kick-sampling cobble substrates at four sites; one upstream and three downstream of the plant outfall (Fig. 1a). Hydropsychidae larvae were flash frozen in liquid nitrogen in the field before being lyophilized, ground and prepared for NMR analysis in the lab. All tissues were analyzed on a Bruker Avance 600 MHz spectrometer using a 5mm cryoprobe to generate ^{1}H NMR spectra.

Principal components analysis (PCA) showed separation in the metabolome among sampling locations. Subsequent partial least squares discriminant analysis (PLS-DA) revealed that the metabolomes of Hydropsychidae larvae from the Meewasin Park and St. Laurent Ferry crossing could not be distinguished. However, metabolomes from the Clarksboro Ferry and Hague Ferry crossing sites were different from each other, as well as different from the Meewasin Park and St. Laurent Ferry crossing sites (Fig. 1b). VIP (Variable Influence on Projection) scores from the PLS-DA indicated that the metabolites alanine, threonine, valine, ATP-ADP, glucose and leucine were important in separating the sampling locations with Meewasin Park and St. Laurent Ferry crossing having similar levels of alanine (Fig. 1c) and other metabolites.

Table 1: Comparison of water chemistry parameters along the South Saskatchewan River at locations of Hydropsychidae larvae collection.

Study Site	MP	CF	HF	SLF
Distance downstream (km)	2	24	51	103
Water temperature (°C)	3.7	1.1	1.9	0.9
Specific Conductivity (µs/cm²)	291	299	297	295
pH	7.99	7.92	7.92	8.21
DO (mg/L)	12.7	17.2	15.1	15.1
TN (µg/L)	486	614	593	507
TP (µg/L)	27	20	26	20

This assessment of ecological condition in the South Saskatchewan River indicates that the effluent from the wastewater treatment plant is affecting ecological status at the Clarksboro Ferry and Hague Ferry crossings. However, ecological conditions show signs of recovery with downstream distance, as the Hydropsychidae metabolome at the St. Laurent Ferry site was not different from the reference site at Meewasin Park. Furthermore, our findings suggest that the sewage effluent is impacting the metabolome through alterations of energy pathways, which is more likely to be a result of exposure to PCPP's rather than nutrients. I recommend further monitoring of the Hydropsychidae metabolome in concert with sampling of PCPP's to establish possible causal effects.

Saskatoon, City of. (2018). Wastewater Treatment Plant. Retrieved from https://www.saskatoon.ca/services-residents/power-water-sewer/sanitary-sewer/wastewater-treatment-plant

Statistics Canada. 2017. Saskatoon [Population centre], Saskatchewan and Manitoba [Province] (table). Census Profile. 2016 Census. Statistics Canada Catalogue no. 98-316-X2016001. Ottawa. Released November 29, 2017. https://www12.statcan.gc.ca/census-recensement/2016/dp-pd/prof/index.cfm?Lang=E (accessed August 20, 2018).

As a bioindicator the metabolome could thus be rapidly adopted into existing Reference Condition Approach based monitoring programs by comparing the metabolome of organisms at test sites to those of reference sites. Recommendations for the operationalization of the metabolome for use in status assessments include: 1) additional research to identify effective indicator species; 2) characterization of inherent biological, spatial, and temporal variation in the metabolome at reference sites; and 3) development of effective monitoring protocols; and 4) generation of assessment models (e.g. reference condition models) (Biales Adam et al., 2015; Campillo et al., 2015).

In addition to being useful for status assessments, the metabolome is also increasingly being used for a variety of other monitoring objectives. Such objectives include environmental effects monitoring of ecosystem impacts, where the metabolome is often employed in a bioassay framework. An example of this is a study by Fasulo et al. (2012), who compared the metabolome of caged mussels in reference and impacted sites, finding differences in the metabolome between the two sites. The metabolome has also been applied towards the identification of priority pollutants at an ecosystem scale. Davis et al. (2016) conducted a study of fathead minnow in the Great Lakes basin where 86 environmental variables were co-analyzed with the metabolomes of exposed fish, ultimately revealing that only 49 of these contaminants showed covariation with the metabolome. The metabolome should therefore be considered for environmental protection capacities in addition to routine monitoring.

Many current bioindicators are limited in their ability to diagnose causes of ecological change. The metabolome has the potential to be used for causal diagnosis because it is predicted that stressors will generate unique patterns of change in the metabolome. Utilization of the metabolome for causal diagnosis thus requires studies to identify associations between individual stressors and the consequent metabolomic shifts. Indeed, metabolomic data is well suited for incorporation into adverse outcome pathways frameworks (AOPs), as AOPs map a stressor response from a molecular initiating event (e.g. carbon monoxide binding haemoglobin), through its metabolomic shift, to its culminating event at a population level (e.g. reduction in abundance) (Ankley et al., 2010; Kramer et al., 2011; Lee et al., 2015; Patlewicz et al., 2015). To date, there are few complete AOPs encompassing a small number of species and stressors, limiting their diagnostic power within a biomonitoring framework due to the potential of similar responses from multiple stressors. However, increased uptake of metabolomics in biomonitoring will broaden the

range of species and stressors for which AOPs exist, and thus enhance confidence in causal assessments through elucidation of unique stressor specific responses.

Despite the strong promise of the metabolome as a biomonitoring tool, we perceive two main impediments to operationalization within mainstream biomonitoring. First, organizations will need to build the capacity for the use of metabolomics. To accomplish this, managers and scientists must be trained or hired to increase institutional understanding of metabolomic data. This shift has already begun in toxicology-focused institutions, where metabolomic applications have been growing steadily for the last 20 years. As uptake of metabolomic methods increase, understanding and knowledge of the types of data produced will become more commonplace, steadily increasing the ease of implementation. Second, communication of metabolomic results to managers, policy makers, and the public may be challenging as metabolomic investigations involve theoretically complicated concepts not as easily conveyed as currently used population or community data. Thus, scientists will need to evolve simple and effective terminology to communicate metabolomic results to stakeholders. Although communication currently presents a significant challenge, widespread adoption of metabolomics within the biomonitoring community will lead to generation of common terms that can be used for public communication. These constraints to operationalization, however, do not represent a lack of scientific capability of the method, but do represent a lack of institutional capacity that will need to develop in order to incorporate new methods.

2.5 Conclusions

Ultimately, current approaches in biomonitoring are unable to integrate all aspects of the biological hierarchy and are therefore incapable of independently meeting all biomonitoring objectives. Our evaluation of the metabolome as an indicator demonstrates that the metabolome meets or has the potential to meet all evaluated criteria of an ideal bioindicator. Moreover, the metabolome has the potential to be used for diagnostic assessment, a benefit not realized by many currently used indicators. Future research identifying stressor specific responses in the metabolome will aid in achievement of diagnostic capacities. Based on our evaluation, we recommend agencies begin to explore the incorporation of the metabolome into their existing ecosystem assessment frameworks. Such incorporation is not without difficulties, as the complete

integration of metabolomics into current biomonitoring frameworks would require an institutional shift in the capabilities of core research groups, however, such shifts are not unprecedented and should not discourage adoption. Adoption of the metabolome will allow organizations to not only assess current ecosystem condition, but also place them at the forefront of the evolution of biomonitoring as metabolomics realizes its intrinsic potential for early warning and causal assessments.

2.6 References

Adams, S. M., & Greeley, M. S. (2000). Ecotoxicological indicators of water quality: Using multi-response indicators to assess the health of aquatic ecosystems. *Water, Air, and Soil Pollution, 123*(1), 103-115. doi:10.1023/A:1005217622959

Adams, S. M., Shepard, K. L., Greeley, M. S., Jimenez, B. D., Ryon, M. G., Shugart, L. R., . . . Hinton, D. E. (1989). The use of bioindicators for assessing the effects of pollutant stress on fish. *Marine Environmental Research, 28*(1-4), 459-464. doi:10.1016/0141-1136(89)90284-5

Ankley, G. T., Bennett, R. S., Erickson, R. J., Hoff, D. J., Hornung, M. W., Johnson, R. D., . . . Villeneuve, D. L. (2010). Adverse outcome pathways: A conceptual framework to support ecotoxicology research and risk assessment. *Environmental Toxicology and Chemistry, 29*(3), 730-741. doi:10.1002/etc.34

Bahamonde, P. A., Feswick, A., Isaacs, M. A., Munkittrick, K. R., & Martyniuk, C. J. (2016). Defining the role of omics in assessing ecosystem health: Perspectives from the Canadian environmental monitoring program. *Environmental Toxicology and Chemistry, 35*(1), 20-35. doi:10.1002/etc.3218

Beckonert, O., Keun, H. C., Ebbels, T. M., Bundy, J., Holmes, E., Lindon, J. C., & Nicholson, J. K. (2007). Metabolic profiling, metabolomic and metabonomic procedures for NMR spectroscopy of urine, plasma, serum and tissue extracts. *Nature protocols, 2*(11), 2692.

Bencic, D. C. (2015). The Challenge: Real-world application of ‘omics endpoints. *Environmental Toxicology and Chemistry, 34*(4), 700-700. doi:10.1002/etc.2839

Biales Adam, D., Fritsch Erika, B., & Connon Richard, E. (2015). In Response: Integration of ‘omics to larger-scale watershed assessments: A mixed government/academic perspective. *Environmental Toxicology and Chemistry, 34*(4), 700-702. doi:10.1002/etc.2841

Bonada, N., Prat, N., Resh, V. H., & Statzner, B. (2006). Developments in aquatic insect biomonitoring: a comparative analysis of recent approaches. *Annu. Rev. Entomol., 51*, 495-523.

Brown, J. H., Gillooly, J. F., Allen, A. P., Savage, V. M., & West, G. B. (2004). Toward a metabolic theory of ecology. *Ecology, 85*(7), 1771-1789. doi:10.1890/03-9000

Brua, R., Culp, J., & Alexander, A. (2015). *Assessment-Specific Field Study Designs and Methods*.

Bundy, J. G., Davey, M. P., & Viant, M. R. (2008). Environmental metabolomics: a critical review and future perspectives. *Metabolomics, 5*(1), 3. doi:10.1007/s11306-008-0152-0

Cairns, J., McCormick, P. V., & Niederlehner, B. R. (1993). A proposed framework for developing indicators of ecosystem health. *Hydrobiologia, 263*(1), 1-44. doi:10.1007/BF00006084

Campillo, J. A., Sevilla, A., Albentosa, M., Bernal, C., Lozano, A. B., Canovas, M., & Leon, V. M. (2015). Metabolomic responses in caged clams, Ruditapes decussatus, exposed to agricultural and urban inputs in a Mediterranean coastal lagoon (Mar Menor, SE Spain). *Science of the Total Environment, 524*, 136-147. doi:10.1016/j.scitotenv.2015.03.136

Canada, E. (2012). *Canadian Aquatic Biomonitoring Network Field Manual Wadeable Streams*. Dartmouth, Nova Scotia.

Cappello, T., Mauceri, A., Corsaro, C., Maisano, M., Parrino, V., Lo Paro, G., . . . Fasulo, S. (2013). Impact of environmental pollution on caged mussels Mytilus galloprovincialis using NMR-based metabolomics. *Marine Pollution Bulletin, 77*(1), 132-139. doi:https://doi.org/10.1016/j.marpolbul.2013.10.019

Connon, R. E., Geist, J., & Werner, I. (2012). Effect-Based Tools for Monitoring and Predicting the Ecotoxicological Effects of Chemicals in the Aquatic Environment. *Sensors (Basel, Switzerland), 12*(9), 12741-12771. doi:10.3390/s120912741

Fasulo, S., Iacono, F., Cappello, T., Corsaro, C., Maisano, M., D'Agata, A., . . . Mauceri, A. (2012). Metabolomic investigation of Mytilus galloprovincialis (Lamarck 1819) caged in

aquatic environments. *Ecotoxicology and Environmental Safety, 84*, 139-146. doi:https://doi.org/10.1016/j.ecoenv.2012.07.001

Gago-Tinoco, A., González-Domínguez, R., García-Barrera, T., Blasco-Moreno, J., Bebianno, M. J., & Gómez-Ariza, J.-L. (2014). Metabolic signatures associated with environmental pollution by metals in Doñana National Park using P. clarkii as bioindicator. *Environmental Science and Pollution Research, 21*(23), 13315-13323. doi:10.1007/s11356-014-2741-y

Gowda, G. A. N., & Djukovic, D. (2014). Overview of mass spectrometry-based metabolomics: opportunities and challenges. *Methods in molecular biology (Clifton, N.J.), 1198*, 3-12. doi:10.1007/978-1-4939-1258-2_1

Hines, A., Oladiran, G. S., Bignell, J. P., Stentiford, G. D., & Viant, M. R. (2007). Direct sampling of organisms from the field and knowledge of their phenotype: key recommendations for environmental metabolomics. *Environmental Science & Technology, 41*(9), 3375-3381.

Hines, A., Staff, F. J., Widdows, J., Compton, R. M., Falciani, F., & Viant, M. R. (2010). Discovery of metabolic signatures for predicting whole organism toxicology. *Toxicological Sciences, 115*(2), 369-378. doi:10.1093/toxsci/kfq004

Hu, M.-Y., Ye, Y.-F., Xue, L.-Y., & Tang, Z.-Y. (2015). Tissue-specific metabolic responses of Cyprinus flammans to copper. *Archives of Environmental Contamination and Toxicology, 69*(1), 112-122. doi:10.1007/s00244-015-0149-4

Ji, C., Li, F., Wang, Q., Zhao, J., Sun, Z., & Wu, H. (2016). An integrated proteomic and metabolomic study on the gender-specific responses of mussels Mytilus galloprovincialis to tetrabromobisphenol A (TBBPA). *Chemosphere, 144*, 527-539.

Ji, C., Wu, H., Wei, L., Zhao, J., & Yu, J. (2013). Proteomic and metabolomic analysis reveal gender-specific responses of mussel Mytilus galloprovincialis to 2,2',4,4'-tetrabromodiphenyl ether (BDE 47). *AQUATIC TOXICOLOGY, 140-141*, 449-457. doi:10.1016/j.aquatox.2013.07.009

Katsiadaki, I., Williams, T. D., Ball, J. S., Bean, T. P., Sanders, M. B., Wu, H., . . . Chipman, J. K. (2010). Hepatic transcriptomic and metabolomic responses in the Stickleback (Gasterosteus aculeatus) exposed to ethinyl-estradiol. *AQUATIC TOXICOLOGY, 97*(3), 174-187. doi:http://doi.org/10.1016/j.aquatox.2009.07.005

Khan, S. A., Liu, X., Li, H., Zhu, Y., Fan, W., Zhou, P., & Rehman, Z. u. (2016). 1H NMR-based serum metabolic profiling of Carassius auratus gibelio under the toxicity of Pb2+ and Cd2. *International Journal of Environmental Science and Technology, 13*(11), 2597-2608. doi:10.1007/s13762-016-1086-0

Kovacevic, V., Simpson, A. J., & Simpson, M. J. (2016). 1H NMR-based metabolomics of Daphnia magna responses after sub-lethal exposure to triclosan, carbamazepine and ibuprofen. *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics, 19*, 199-210. doi:https://doi.org/10.1016/j.cbd.2016.01.004

Kramer, V. J., Etterson, M. A., Hecker, M., Murphy, C. A., Roesijadi, G., Spade, D. J., . . . Ankley, G. T. (2011). Adverse outcome pathways and ecological risk assessment: Bridging to population-level effects. *Environmental Toxicology and Chemistry, 30*(1), 64-76. doi:10.1002/etc.375

Lankadurai, B. P., Nagato, E. G., & Simpson, M. J. (2013). Environmental metabolomics: an emerging approach to study organism responses to environmental stressors. *Environmental Reviews, 21*, 180+. Retrieved from http://go.galegroup.com.proxy1.lib.uwo.ca/ps/i.do?p=AONE&sw=w&u=lond95336&v=2.1&it=r&id=GALE%7CA351613219&sid=summon&asid=f0dda50f889f09742b185ae9690b07ef

Lee, J. W., Won, E.-J., Raisuddin, S., & Lee, J.-S. (2015). Significance of adverse outcome pathways in biomarker-based environmental risk assessment in aquatic organisms. *Journal of Environmental Sciences, 35*, 115-127.

Li, M. H., Wang, J. S., Lu, Z. G., Wei, D. D., Yang, M. H., & Kong, L. Y. (2014). NMR-based metabolomics approach to study the toxicity of lambda-cyhalothrin to goldfish (Carassius auratus). *AQUATIC TOXICOLOGY, 146*, 82-92. doi:10.1016/j.aquatox.2013.10.024

Lin, C. Y., Anderson, B. S., Phillips, B. M., Peng, A. C., Clark, S., Voorhees, J., . . . Todd, C. R. (2009). Characterization of the metabolic actions of crude versus dispersed oil in salmon smolts via NMR-based metabolomics. *AQUATIC TOXICOLOGY, 95*(3), 230-238.

Lin, C. Y., Wu, H., Tjeerdema, R. S., & Viant, M. R. (2007). Evaluation of metabolite extraction strategies from tissue samples using NMR metabolomics. *Metabolomics, 3*(1), 55-67.

Lu, J., Feng, J., Cai, S., & Chen, Z. (2017). Metabolomic responses of Haliotis diversicolor to organotin compounds. *Chemosphere, 168*, 860-869.

Martyniuk, C. J. (2018). Are we closer to the vision? A proposed framework for incorporating omics into environmental assessments. *Environmental toxicology and pharmacology, 59*, 87-93. doi:https://doi.org/10.1016/j.etap.2018.03.005

Miller, M. G. (2007). Environmental metabolomics: a SWOT analysis (strengths, weaknesses, opportunities, and threats). *Journal of proteome research, 6*(2), 540-545.

Morrison, N., Bearden, D., Bundy, J. G., Collette, T., Currie, F., Davey, M. P., . . . Viant, M. R. (2007). Standard reporting requirements for biological samples in metabolomics experiments: environmental context. *Metabolomics, 3*(3), 203-210. doi:10.1007/s11306-007-0067-1

Munkittrick, K. R., & McCarty, L. S. (1995). An integrated approach to aquatic ecosystem health: top-down, bottom-up or middle-out? *Journal of Aquatic Ecosystem Health, 4*(2), 77-90. doi:10.1007/BF00044791

Pan, Z., & Raftery, D. (2007). Comparing and combining NMR spectroscopy and mass spectrometry in metabolomics. *Analytical and Bioanalytical Chemistry, 387*(2), 525-527. doi:10.1007/s00216-006-0687-8

Patlewicz, G., Simon, T. W., Rowlands, J. C., Budinsky, R. A., & Becker, R. A. (2015). Proposing a scientific confidence framework to help support the application of adverse outcome pathways for regulatory purposes. *Regulatory Toxicology and Pharmacology, 71*(3), 463-477. doi:http://dx.doi.org/10.1016/j.yrtph.2015.02.011

Peng, B., Li, H., & Peng, X.-X. (2015). Functional metabolomics: from biomarker discovery to metabolome reprogramming. *Protein & cell, 6*(9), 628-637. doi:10.1007/s13238-015-0185-x

Peñuelas, J., & Sardans, J. (2009a). Ecological metabolomics. *Chemistry and Ecology, 25*(4), 305-309. doi:10.1080/02757540903062517

Peñuelas, J., & Sardans, J. (2009b). Elementary factors. *Nature, 460*, 803. doi:10.1038/460803a

Qiao, Q., Le Manach, S., Sotton, B., Huet, H., Duvernois-Berthet, E., Paris, A., . . . Marie, B. (2016). Deep sexual dimorphism in adult medaka fish liver highlighted by multi-omic approach. *Scientific Reports, 6*, 32459. doi:10.1038/srep32459

https://www.nature.com/articles/srep32459#supplementary-information

Reece, P. F., & Richardson, J. S. (1999). *Biomonitoring with the reference condition approach for the detection of aquatic ecosystems at risk.* Paper presented at the Proceedings of a Conference on the Biology and Management of species and habitats at risk.

Santos, E. M., Ball, J. S., Williams, T. D., Wu, H., Ortega, F., van Aerle, R., . . . Tyler, C. R. (2010). Identifying health impacts of exposure to copper using transcriptomics and metabolomics in a fish model. *Environmental Science & Technology, 44*(2), 820-826. doi:10.1021/es902558k

Simmons Denina, B. D., Benskin Jonathan, P., Cosgrove John, R., Duncker Bernard, P., Ekman Drew, R., Martyniuk Christopher, J., & Sherry James, P. (2015). Omics for aquatic ecotoxicology: Control of extraneous variability to enhance the analysis of environmental effects. *Environmental Toxicology and Chemistry, 34*(8), 1693-1704. doi:10.1002/etc.3002

Skelton, D. M., Ekman, D. R., Martinović-Weigelt, D., Ankley, G. T., Villeneuve, D. L., Teng, Q., & Collette, T. W. (2014). Metabolomics for in situ environmental monitoring of surface waters impacted by contaminants from both point and nonpoint sources. *Environmental Science & Technology, 48*(4), 2395-2403. doi:10.1021/es404021f

Stearns, S. C. (1989). Trade-offs in life-history evolution. *Functional Ecology, 3*(3), 259-268. doi:10.2307/2389364

Taylor, N., Gavin, A., & Viant, M. (2018). Metabolomics discovers early-response metabolic biomarkers that can predict chronic reproductive fitness in individual Daphnia magna. *Metabolites, 8*(3). doi:10.3390/metabo8030042

Viant, M. R. (2007). Metabolomics of aquatic organisms: the new 'omics' on the block. *Marine Ecology Progress Series, 332*, 301-306. Retrieved from http://www.int-res.com/abstracts/meps/v332/p301-306/

Viant, M. R. (2008). Recent developments in environmental metabolomics. *Molecular BioSystems, 4*(10), 980-986. doi:10.1039/B805354E

Wagner, N. D., Simpson, A. J., & Simpson, M. J. (2017). Metabolomic responses to sublethal contaminant exposure in neonate and adult Daphnia magna. *Environmental Toxicology and Chemistry, 36*(4), 938-946. doi:10.1002/etc.3604

Wu, H., Southam, A. D., Hines, A., & Viant, M. R. (2008). High-throughput tissue extraction protocol for NMR- and MS-based metabolomics. *Analytical Biochemistry, 372*(2), 204-212. doi:https://doi.org/10.1016/j.ab.2007.10.002

Wu, H., Xu, L., Yu, D., & Ji, C. (2017). Differential metabolic responses in three life stages of mussels Mytilus galloprovincialis exposed to cadmium. *Ecotoxicology, 26*(1), 74-80. doi:10.1007/s10646-016-1741-8

Chapter 3

3 Application of Metabolomics to Testing of Sublethal Effects of Naphthenic Acids on *Hexagenia spp.*

3.1 Introduction

The Athabasca Oil Sands region in Alberta, Canada has been an area of environmental concern since the commencement of bitumen extraction operations in the late 1960's (Humphries, 2008). A potential source of environmental contamination arising from bitumen extraction are Oil Sands Process Waters (OSPWs) (Giesy et al., 2010). Regulations currently prohibit release of OSPWs as they contain several environmental contaminants including polyaromatic hydrocarbons, heavy metals, and naphthenic acids. However, the large amassment of OSPW in tailings ponds is leading to increasing pressure from industry to allow release of OSPW to adjacent waterways. Research is thus needed to discern potential environmental effects of exposure to individual OSPW components.

Naphthenic acids (NAs) have been hypothesized to contribute significantly to OSPW toxicity due to their surfactant properties (Frank et al., 2008). NAs are classically defined as a mixture of saturated hydrocarbons containing both a non-aromatic cyclic component and a carboxylic acid functional component (Headley et al., 2009; Headley et al., 2007; Hsu et al., 2000). Although industrial samples often contain a significant proportion of heteroatoms (i.e. sulfur or nitrogen containing NAs), NA samples are most commonly dominated by species with a single carboxylic acid functional group (Marentette et al., 2015). As much of the research on NAs has focused on larger mixtures of these structural components, it is imperative that toxicity of individual components be elucidated to allow for development of effective toxicity mitigation strategies and prediction of environmental impacts.

Although little literature exists on the environmental fate of NAs, it is known that NAs sorb to organic matter in sediment (Janfada et al., 2006). When selecting an organism to act as an indicator of adverse ecological effects of NA exposure, it therefore follows that the organism should have interaction with sediment organic matter. *Hexagenia spp*. is a group of mayflies (Ephemeroptera) that are prevalent across North America, including the Athabasca Oils Sands region. The larvae of *Hexagenia spp*. are burrowing insects that ingest organic material, and thus

are likely to be directly exposed to NAs in the environment. In addition, these organisms represent a key species within the ecosystem due to their function as a significant food source for many other organisms and as such the loss of this species could have significant impacts on aquatic communities.

Impacts of NAs on the ecology of aquatic ecosystems have been shown in community composition of phytoplankton (Leung et al., 2003) and bacteria (Hadwin et al., 2006). To my knowledge shifts in benthic invertebrate community structure as a result of NA exposure have not yet been assessed despite being a common endpoint for monitoring. However, due to the intrinsic nature of community level assessments, changes in community composition are only visible after the effect of the contaminant has already impacted the community and therefore have the potential to be negative and irreversible. In contrast, the an organism's metabolome could function as an early warning indicator of NA stress by highlighting stress within organisms before it is observable on a community scale.

The metabolome of an organism is an indicator that captures the summation of all small molecule metabolites, and serves as surrogate for the physiological status of an organism. Although the metabolome represents the physiological state of an organism, changes in the metabolome have been previously shown to correlate to observable changes in fitness (Taylor et al., 2018). Therefore, a change in the metabolome can be indicative of future ecological changes, which would propagate up the ecological hierarchy (e.g., population and community levels) as a result. As such, the metabolome of *Hexagenia spp.* may have the potential to serve as an early response and ecologically relevant indicator in the detection of environmental effects of NA exposure.

3.2 Research Questions and Objectives

In response to the need for knowledge surrounding toxicity of NAs to aquatic organisms, I evaluated a fitness related bioindicator of NA exposure for use in ecological monitoring of NA effects in aquatic ecosystems. I related changes in the metabolome and in fitness of the larval stage of a common aquatic insect, *Hexagenia spp.*, to a gradient of NA concentrations to determine ecologically relevant toxicity thresholds of NA exposure. Additionally, by relating the metabolomic profile to the overall fitness of *Hexagenia spp.* the knowledge gained from this study

contributes to predictions of potential population level effects. To achieve this goal, I answered the following two questions:

1. Is there an association between the concentration of NAs in water and the metabolome of *Hexagenia spp.*?

Prediction: The concentration of NAs in the stream environment will be associated with changes in the metabolome of *Hexagenia spp.*. Current knowledge regarding NA toxicity supports a narcotic mode of action (Frank et al., 2009) and therefore it is also predicted that the observed change in metabolome will exhibit increases in metabolites associated with cell narcosis. Additionally, based on observed sublethal changes in other aquatic species, it is predicted that metabolites associated with endocrine disruption, oxidative stress, and disruption to energy metabolism pathways will see change in expression though examination of the metabolome (Marentette et al., 2017; Melvin et al., 2013; Wang et al., 2015).

2. Are observed changes in metabolome of *Hexagenia spp.* correlated with the observed fitness (i.e., growth) of the organism?

Prediction: Mayflies exposed to greater naphthenic acid concentrations will have a lower overall growth rate compared to those in the control treatment. This prediction is supported by the finding that tadpoles showed reduced growth when exposed to sublethal concentrations of naphthenic acids (Melvin et al., 2013). Since cells are expected to undergo stress associated responses, this would suggest that more resources will be required to maintain normal body function through the replacement of affected cells, and therefore less energy would be available for growth in individuals exposed to NAs and these individuals will have to rely on previous energy stores for survival.

3.3 Methods

3.3.1 *Hexagenia spp.* Collection and Culture

Hexagenia nymphs used in this experiment were cultured from eggs collected in June 2016 at the mouth of Lake St. Clair (42° 20' 22.1" N 82° 55' 51.6" W), near Windsor, Ontario, Canada. Eggs were collected in the evening by manually collecting sexually mature females. Females were removed from outdoor surfaces and placed in a large, plastic Ziploc™ bag containing culture water (dechlorinated tap water (Burlington, Ontario)) and a pinch of facial clay to prevent eggs from clumping together. Bags were shaken to allow the eggs to dislodge from the bodies of the females. Thirty individuals were placed in each plastic bag and allowed to remain in the water at room temperature overnight. The following day, female carcasses were removed from the bags and the bags were resealed. Eggs were stored in culture water at room temperature for 6 days, followed by 14 °C for 6 days, and eventually at 4 °C for a maximum of one year before use.

Hexagenia were cultured at the Environment and Climate Change Canada laboratory in Burlington, Ontario, Canada. Culture tanks were washed with Extran, 20 % HCl, and thoroughly rinsed prior to use. Culture sediment (7.3 % TOC) was composed of sediment collected in June 2016 from the Long Point Marsh and stored at 4 °C. Prior to use, sediment was filtered through a 250 µm sieve to homogenize sediment and remove any extraneous biological material. Each culture tank was lined with approximately 2 cm of sediment, filled with culture water, and warmed to 24 °C. Twenty milliliters of food mixture (4 g ground Tetramin™, 3 g yeast, and 3 g cereal grass in 100 mL distilled water) was added to each tank 24 hours prior to animal addition. Three hundred hatched individuals were placed in each tank, and allowed to settle before gentle aeration began 1 hour later. Cultures were fed weekly, each culture tank receiving approximately 0.8 g of food mixture every 7 d. Individual *Hexagenia* were 6-8 weeks of age at experiment start. Ages of individuals were randomized among treatments.

3.3.2 Sodium Naphthenate Characterization

Sodium naphthenate was purchased from TCI Chemicals America (Portland, Oregon, USA) and characterized by mass spectrometry. Analyses found the purchased naphthenate mixture to contain 99.8% O_2 species, with the remaining 0.2% comprised of S_5 species. The sample ranged in carbon number from 8 - 22, with 1.5 - 7.5 O_2 DBE (Fig. 2). The sample also contained a significant amount of octanoic acid relative to other species (more than 2.5 times the relative intensity of the next greatest species).

Experimental concentrations of sodium naphthenate were generated by a process of serial dilutions. Sodium naphthenate was first dissolved to create a stock solution of verified concentration (55.73 ± 5.18 (SD) mg Na-NA / L). Stock solution was added to microcosm tanks to achieve desired concentrations (0 µg/L, 0.0001 µg/L, 0.001 µg/L, 0.006 µg/L, 0.01 µg/L, 0.04 µg/L, 0.08 µg/L, 0.1 µg/L, 0.4 µg/L, 0.8 µg/L, 1 µg/L, 6 µg/L, 10 µg/L, 60 µg/L, 100 µg/L). Concentrations were chosen based on preliminary data of acute naphthenic acid toxicity to mayflies (Unpublished data, Alexander-Trusiak et al.). For low concentrations where one serial dilution would be insufficient to achieve the desired concentration, stock solution was diluted to create a lower concentration stock solution from which precise quantities were measured and added to microcosms.

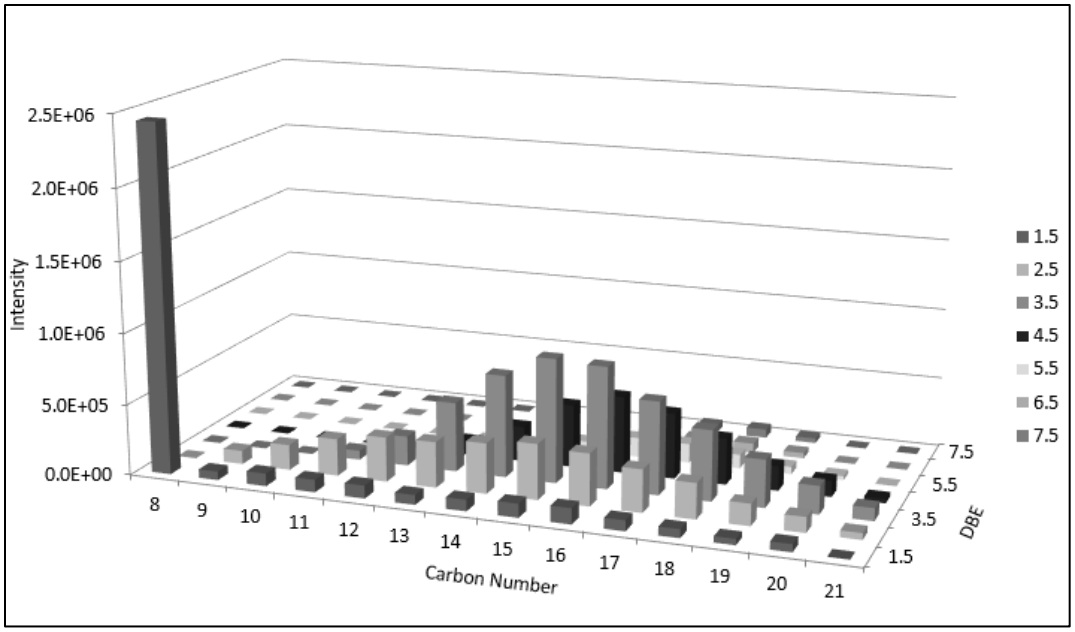

Figure 2: Structural distribution of O_2 sodium naphthenic species in standard sample

3.3.3 Experimental Procedures

Prior to the beginning of the experiment, all microcosms (2 L jars) were washed with Extran and 20% HCl. Microcosm sediment was collected from Long Point, Ontario, Canada in June 2016 and stored at 4 °C and consisted of a mixture of sediments collected from Long Point Marsh and Long Point Bay to achieve a total organic carbon content of 2 %. 250 mL of microcosm sediment was added to each microcosm, followed by the addition of 1.2 L culture water. Sediment was allowed to settle in the microcosm for 24 hours before the addition of specified volumes of sodium naphthenate stock solution. Microcosms were assigned to one of fifteen different concentrations of sodium naphthenate ranging from 0-100 µg/L (0 µg/L, 0.0001 µg/L, 0.001 µg/L, 0.006 µg/L, 0.01 µg/L, 0.04 µg/L, 0.08 µg/L, 0.1 µg/L, 0.4 µg/L, 0.8 µg/L, 1 µg/L, 6 µg/L, 10 µg/L, 60 µg/L, 100 µg/L). Four microcosms were assigned to each treatment concentration (N = 60 microcosms) with 18 mayflies randomly assigned to each microcosm (N = 1080). Post sodium naphthenate addition, microcosms were aerated to >90% dissolved oxygen saturation for 6 days prior to experiment start. After the 6-day period, aeration was stopped to allow addition of 18 mayflies and then restarted 1 hour after addition. Individual mayflies were massed in groups of 9 to allow for growth determinations prior to placement in the randomly assigned microcosm.

Microcosms were placed in a temperature-controlled chamber at 24°C for the duration of the 21-day exposure period. Each microcosm was aerated consistently to ensure adequate dissolved oxygen, and exposed to a 16h:8h light:dark cycle. Microcosms also received 50 mg of culture food mixture once weekly to prevent starvation. Specific conductance ($\mu S/cm^2$), pH, percent dissolved oxygen (DO) and DO concentration (mg/L) was monitored weekly (4 times in total) in each microcosm using a YSI probe.

At the conclusion of the exposure period, each microcosm was removed from the growth chamber and individual mayflies were separated from exposure sediment. The surviving mayflies were counted and massed as a group to allow for mean growth determinations. The surviving individuals were then split into two groups (n = 9,8, or 7 individuals dependent on survival; no significant relationship to treatment level) to ensure sufficient mass for tissue extraction and NMR metabolomics. Each group was placed in a cryotube, flash frozen in liquid nitrogen and stored at -80 °C until tissue extraction.

3.3.4 Tissue Extraction

Whole groups of nine mayflies were used for tissue extraction. Mayflies were lyophilized overnight and subsequently homogenized using a Precellys homogenizer. 10 mg (dry weight) of ground tissue was added to a 2 mL Eppendorf tube. Tissue samples were extracted using a 2:2:1.8 chloroform:methanol:water extraction (Viant 2007). Ice-cold methanol (0.6 ml) and ice-cold Millipore water (0.27 ml) were added to each sample and then vortexed three times for 15 seconds, followed by centrifugation at 13,400 rpm for ten minutes. The supernatant was then collected and transferred to a new Eppendorf vial, where ice-cold chloroform (0.6 ml) and ice-cold Millipore water (0.27 ml) was added. Samples were vortexed for 60 s, allowed to partition on ice for ten minutes, and centrifuged at 13,400 rpm for 10 minutes. The methanol (upper) and chloroform (lower) layers were then removed and placed into separate Eppendorf tubes. Solvents were then evaporated using a Speedvac Concentrator (ThermoScientifc), and subsequently stored at -80 °C until resuspension in appropriate solvent (sodium phosphate buffer for polar metabolites and 2:1 chloroform:methanol mixture for lipophilic metabolites) for NMR analyses.

3.3.5 NMR Spectroscopy

NMR analyses were completed on both the polar and lipophilic fraction of the metabolome. All ^{1}H 1D NMR spectra were acquired at 298 K using a Bruker Avance 600 MHz spectrometer equipped with a 5 mm broadband probe and operating at 600.17 MHz. The instrument was locked to the appropriate deuterium resonance (D_2O for polar metabolites, Methanol-d4 for lipophilic metabolites), tuned, and shimmed prior to each spectral acquisition.

Polar metabolites were resuspended in 0.75 mL 100 mM sodium phosphate buffer (pH = 7.0) with 3 mM NaN_3 added as a preservative. Trimethylsilylpropanoic acid (TMSP) was added to the buffer as an internal standard. Samples were vortexed for 60 s, centrifuged for 5 minutes, and 0.6 mL of resuspended solution was then placed immediately in a 5 mm NMR tube for analysis. An excitation sculpting pulse sequence (Bollard et al., 2005) with a 60° pulse was used

to maximize water suppression. Data were acquired with a relaxation delay of 2 s, spectral width of 9615.385 Hz, and 128 scans. Data were then Fourier transformed, phased, baseline corrected using a polynomial baseline correction, and referenced to TMSP. Spectra were processed with LB = 0.3 and zero filled to 32768 data points.

In addition to standard analysis of the polar metabolome, a second analysis was conducted in order to investigate the role of enzyme denaturation on the metabolome. Schock et al. (2016) have shown that adding a five-minute heating step to the sample preparation regime post resuspension and prior to analysis stabilized the metabolome. Additionally, it was demonstrated that several metabolite abundances were altered during the heating process. This analysis was therefore conducted on samples identical to the non-heated polar analysis in order to uncover any significant metabolite changes potentially overshadowed by increased inter-sample variability.

Lipophilic metabolites were resuspended in 0.75 mL 2:1 chloroform-d:methanol-d_4 mixture containing 0.5 mM tetramethylsilane (TMS) as an internal standard. Samples were vortexed for 60 s, centrifuged for 5 minutes, and 0.6 mL of resuspended solution was then placed immediately in a 5 mm NMR tube for analysis. A standard 90° pulse acquire sequence with a 2.7 s acquisition time and 3 s relaxation delay, 128 scans, and 12019.230 Hz spectral width was used. Data were then Fourier transformed, phased, baseline corrected using a polynomial baseline correction, and referenced to TMS. Spectra were processed with LB = 0.3 and zero filled to 65536 data points. All spectra were integrated and normalized to the area of the standard peak (TMSP or TMS) (Viant, 2003).

3.3.6 Statistical Analysis

Statistical analysis was completed with Systat and MetaboAnalyst software. Prior to binning, spectrally visual outliers were excluded from analysis. Remaining samples were transformed with a general log transformation (Parsons et al. 2007), and binned from -1 to 10 ppm into 0.005 ppm wide bins for data analysis. Following binning, samples were analyzed via PCA by MetaboAnalyst (Xia and Wishart, 2016). PCA scores along component 1 and 2 were then further analyzed in Systat with a General Linear Model to assess significance below p=0.1. If

significant, components were then subjected to Tukey's post-hoc pairwise test to determine significant differences between individual concentrations. Growth and survival data were analyzed in Systat using an ANOVA to assess differences between control and treatment groups.

3.4 Results

3.4.1 Physicochemical Parameters

Measured water parameters were not different between treatments. Water temperature in the microcosms was an average of 22.5 °C (maximum = 22.8 °C, minimum = 21.9 °C). Dissolved oxygen was an average of 101.2%, ranging from 88.4% - 119.2%. Temperature and dissolved oxygen values did not exhibit any trends throughout the duration of the experiment. Specific conductivity was an average of 450.2 $\mu S/cm^2$, ranging from 360.1 $\mu S/cm^2$ to 546.0 $\mu S/cm^2$. Specific conductivity generally increased with time for all treatment levels. pH in the microcosms was an average of 8.17, ranging from 7.92 to 8.37. pH decreased between Day 0 and Day 7 in treatment levels, but did not exhibit any trends in the following two weeks. Overall, there were no trends in water chemistry which distinguished any treatment from the control (Figure 3).

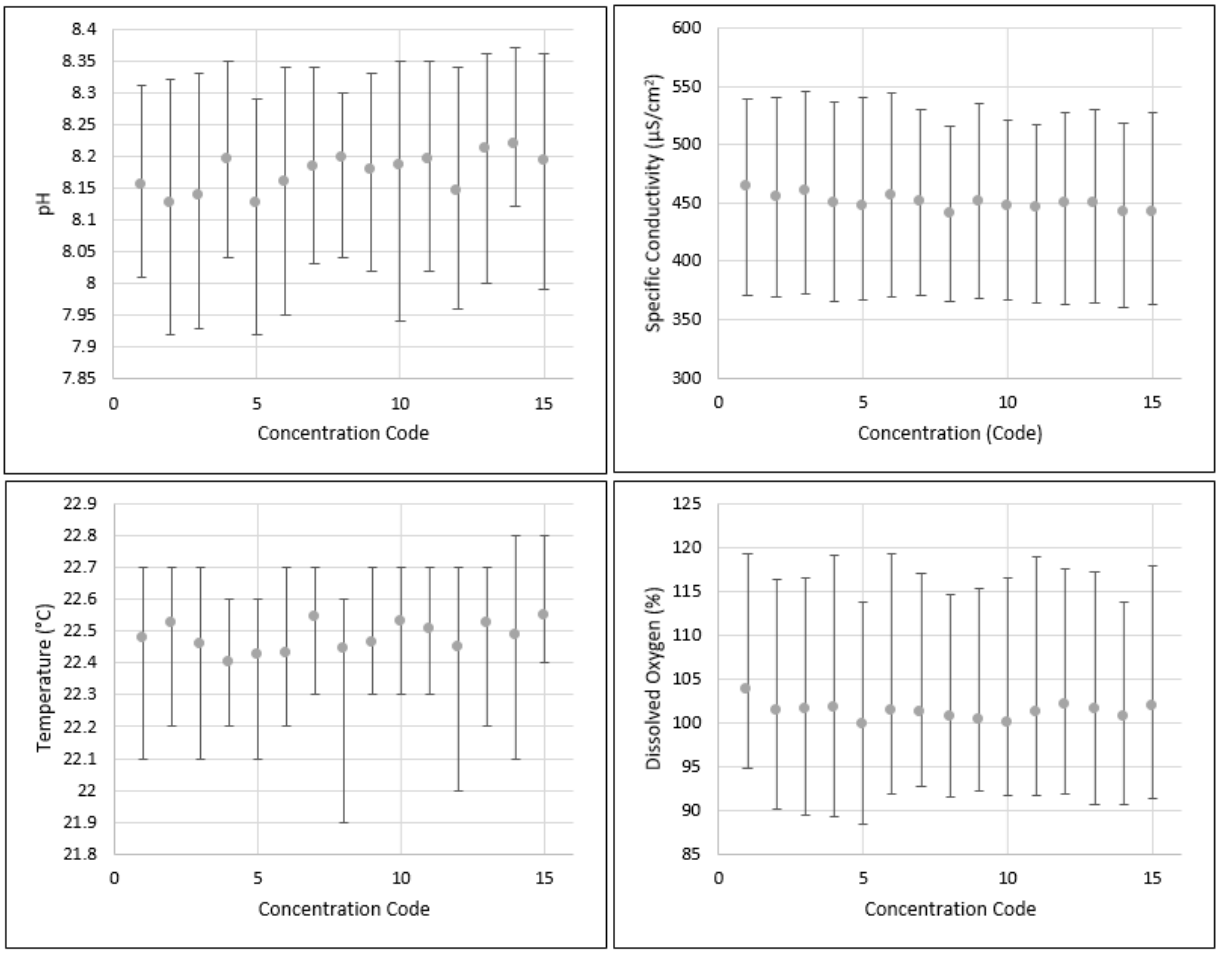

Figure 3: Monitored water chemistry parameters in each of the microcosms throughout the study period: centre point represents the average and error bars represent the range of each parameter; concentration code refers to C1-C15, with C being the control and C15 being 0.1mg/L

3.4.2 Survival and Growth

Individual mayflies weighed an average of 15.92 mg at the beginning of the experiment. Throughout the duration of the exposure, each mayfly gained an average of 1.09 mg per day, resulting in an average mass of 38.78 mg at the end of the experiment. No differences in growth were observed between treatment levels ($p=0.909$) (Figure 5). One-way ANOVA of survival between control and high exposure treatments showed no difference in survival between treatments ($p=0.224$).

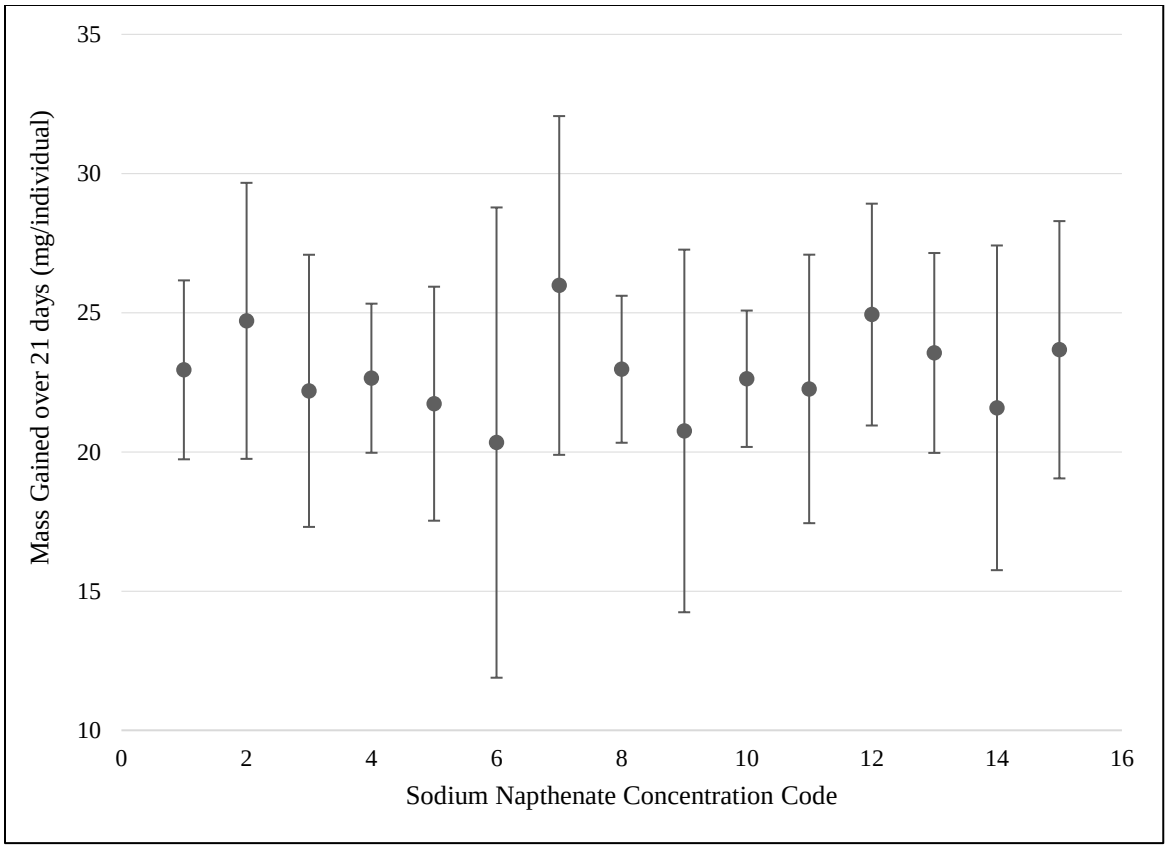

Figure 4: Average *Hexagenia spp.* mass gained (mg / individual) over the 21-d exposure to varying concentrations of sodium naphthenate, dots represent average mass gained and error bars represent standard deviation

3.4.3 Polar Metabolome

Removed outliers for the non-heated aqueous analysis included 3A (C6), 36A (C4), 46B (C8), 11A (C7), 26B (C9), and 25A (C7). No statistically significant separation was observed in the non-heated aqueous metabolome along PC1 ($p=0.125$) or PC2 ($p=0.625$) (Figure 6). Removed outliers for the heated analysis included 5A (C13), 5B (C13), 13A (C11), 13B (C11), 14B (C12), 25A (C7), and 51A (C15). No significant relationships were found along PC1 ($p=0.234$) or PC2 ($p=0.741$) when heated samples were used.

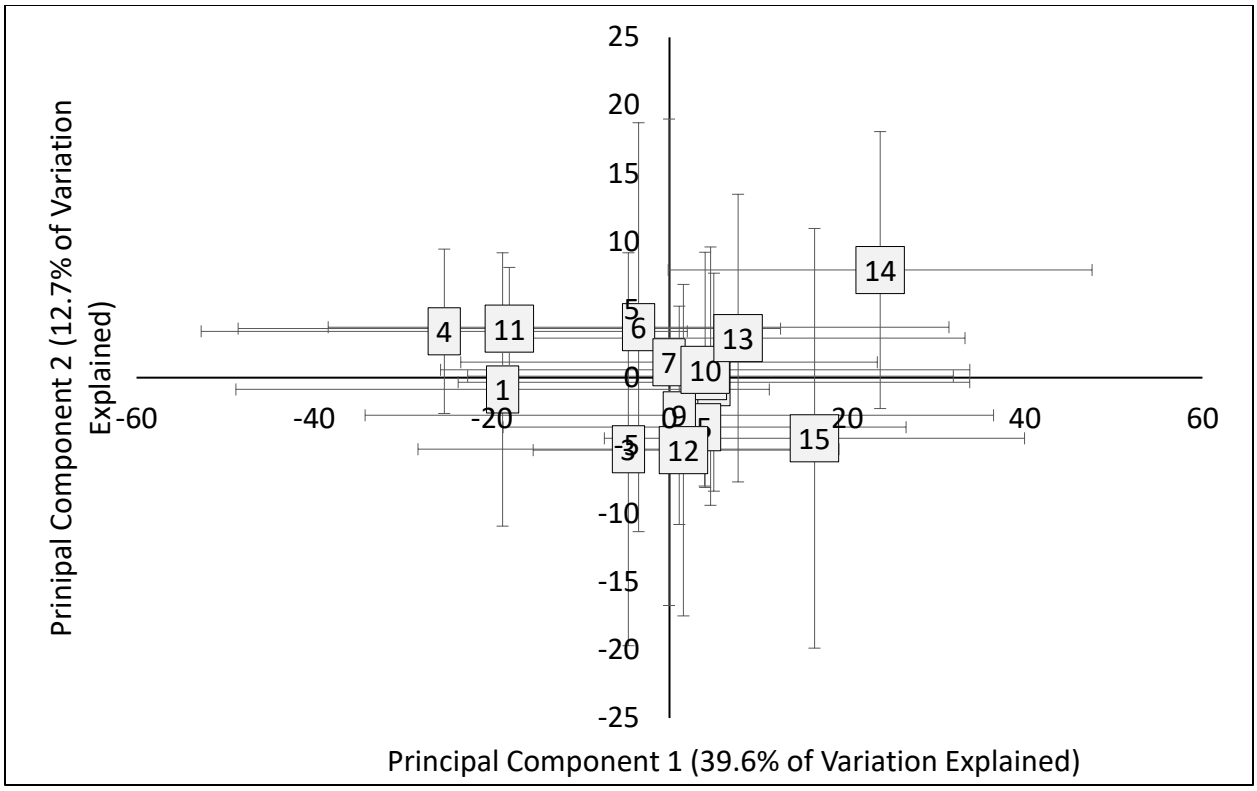

Figure 5: Polar metabolome PCA scores plot utilizing non-heated analysis method; numbers represent exposure concentrations from C1 (0 mg/L) to C15 (0.1 mg/L)

PCA analyses to compare heated and unheated metabolomes revealed almost complete separation of the two sets of metabolomes along PC2 ($p<0.001$), but not PC1 ($p=0.824$) (Figure7). 47% of the total variation was explained along the first two PCs, with the first component explaining 28.5% of variation, and the second component explaining 18% of the variation. PLS-DA analyses revealed complete separation along component 1 ($Q2=0.90$ after 1 component with an accuracy of 98.7%, 0.94 after 2 components with an accuracy of 99.6% when classifying via LOOCV).

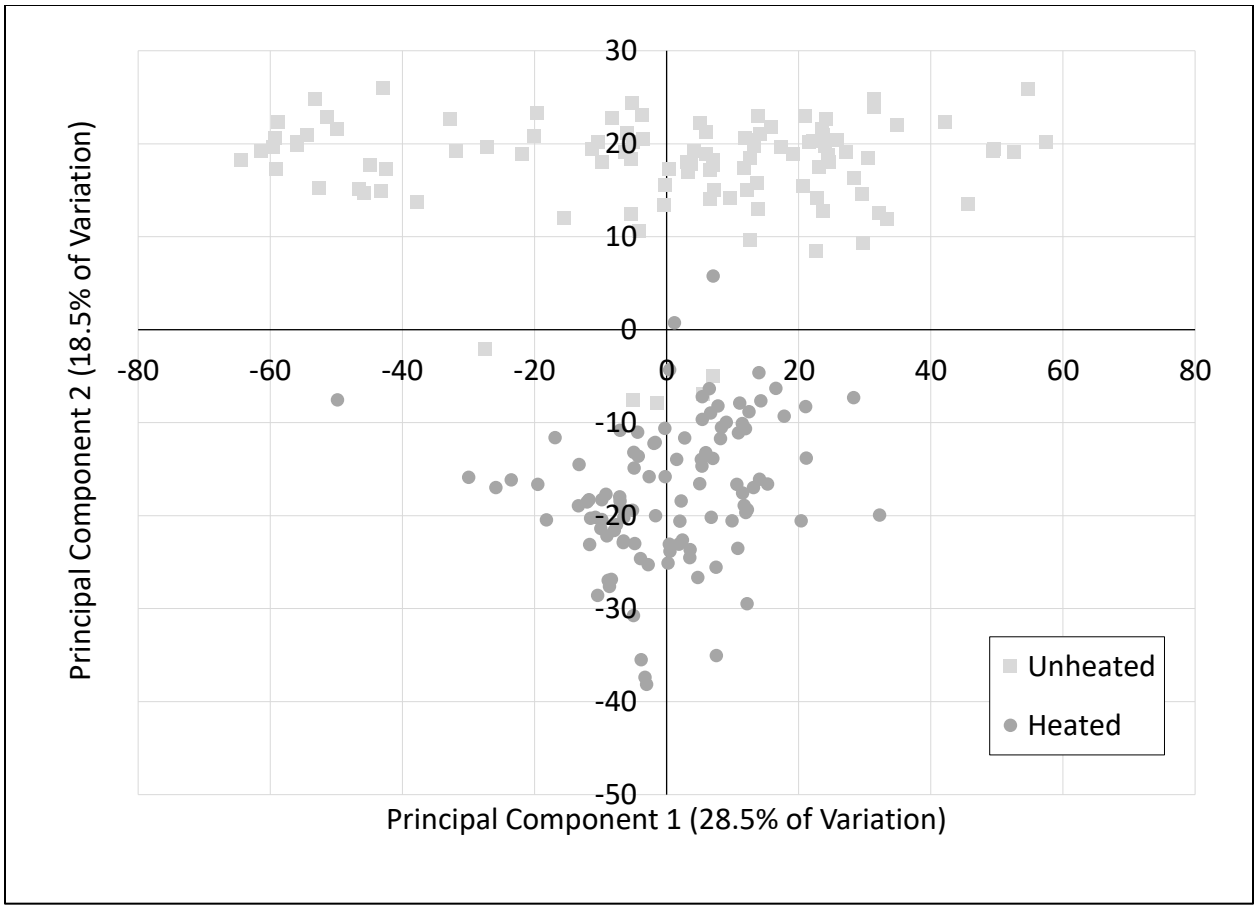

Figure 6: PCA scores plot comparing the heated and unheated polar metabolome

3.4.4 Lipid Metabolome

Outliers removed from lipid analysis include 2A (C9), 7A (C3), 13A (C11), 13B (C11), 14B (C12), and 15A (C10). The first two principal components explained 36.4% of the total variation, with PC1 explaining 28.6% and PC2 explaining 7.8%. General linear model analyses indicated differences among treatment levels in the lipid metabolome along PC2 ($p=0.069$), but not PC1 ($p=0.930$). Tukey's pairwise comparisons show a significant difference between the control and the highest exposure level ($p=0.088$). No other individual concentrations were significantly different from the control or from each other along PC2.

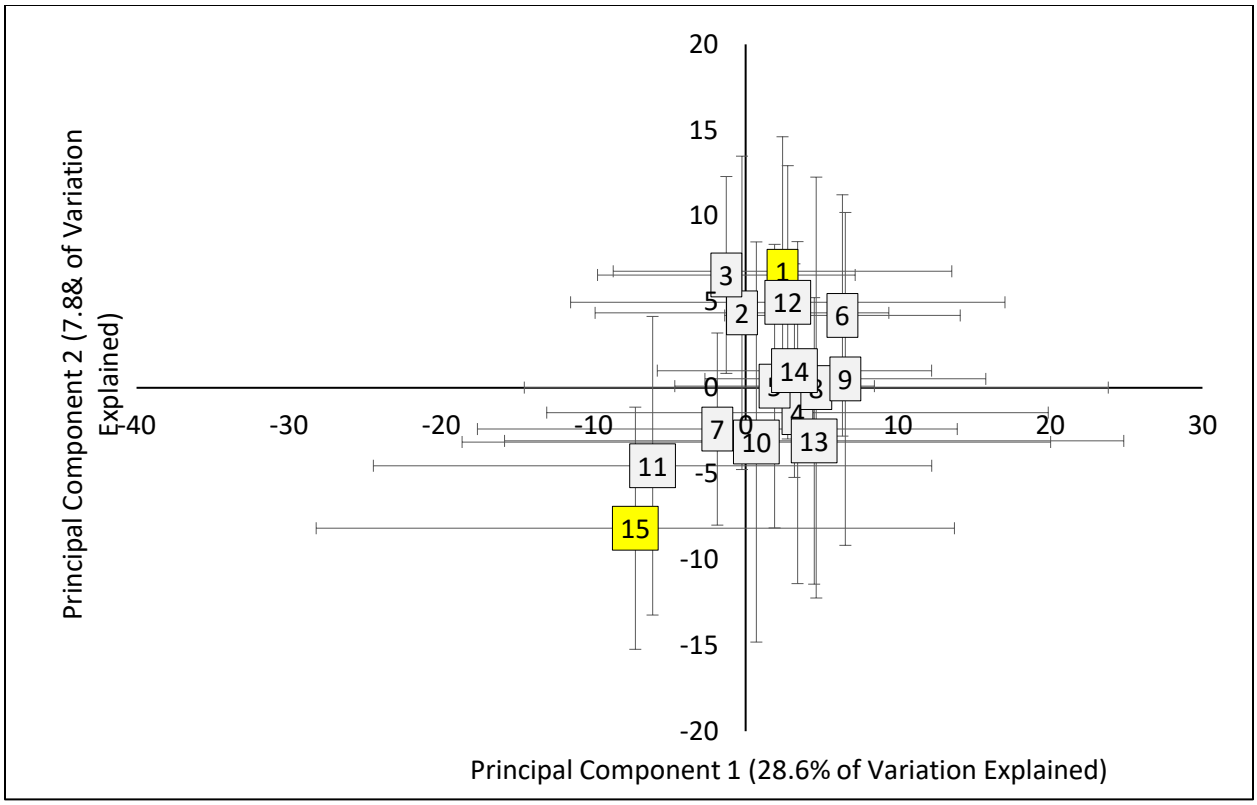

Figure 7: Lipid metabolome PCA scores plot; yellow fill represents treatments which are significantly different from each other; numbers represent exposure concentrations from C1 (0 mg/L) to C15 (0.1 mg/L)

3.5 Discussion

Results of this investigation revealed what could potentially be the beginning of a threshold response of the lipid metabolome of *Hexagenia spp*. to naphthenic acid exposure. Specifically, the lipid metabolome of *Hexagenia spp*. did not respond below a naphthenic acid concentration of 0.1 mg/L. However, exposure to a naphthenic acid concentration of 0.1 mg/L caused a significant change in the metabolome in comparison to the control. The change in the lipid metabolome was observed only at the highest concentration tested suggesting that a stress response may be beginning to occur and therefore a concentration of 0.1 mg/L may represent the beginning of a threshold concentration for initiation of physiological changes. While previously reported concentrations suggest lethal doses for other aquatic organisms at much higher concentrations (0.15 - 56.2 mg/L, (Dokholyan & Magomedov, 1983; Kinley et al., 2016; Swigert et al., 2015)), my finding suggests that physiological changes may begin at substantially lower concentrations.

Despite a change in the lipid metabolome of *Hexagenia spp.* exposed to sublethal naphthenic acid concentrations, a corresponding difference in growth was not observed. An implication of my observation is that, while there is a change in the metabolome, this does not translate to a decrease in observed fitness at the tested concentrations. As such, changes in the metabolome at exposure levels of 0.1 mg/L may not have an ecological consequence. The fact that lipid metabolome has demonstrated a potential threshold response while growth remained unchanged suggests a lag between when effects are visible in the metabolome and when effects have observable ecological consequences. In terms of management, the gap between a metabolomic response and an observable fitness response would allow for the metabolome to serve as an early warning of ecologically relevant effects before they occur. Early warning indicators are critical tools as they increase potential for management actions to be effective at mitigating ecologically relevant effects such as population decline effects. Future research is needed to determine if greater environmental naphthenic acid concentrations have a fitness consequence for mayflies and to elucidate the difference in thresholds between changes in the metabolome and changes in fitness.

In contrast to the lipid metabolome, the polar metabolome of the exposed *Hexagenia spp.* did not respond to a naphthenic acid concentration at or below 0.1 mg/L. Different responses from the lipid and polar metabolomes may be related to the toxic mode of action of naphthenic acids.

Naphthenic acids have been hypothesized to cause membrane disruption due to the surfactant properties of naphthenic acids (Frank et al., 2009). Membrane disruption as a principal toxic mode of action would align with the observed change in the lipid metabolome occurring before the polar metabolome. Membranes are primarily composed of lipid molecules, therefore it follows that the lipid metabolome would be faster responding if membrane disruption is the actual mode of toxic action. Based on this finding the lipid metabolome may be a more effective early warning indicator for monitoring the effects of naphthenic acids on aquatic ecosystems. Moreover, the finding that the lipid and aqueous metabolome react differently to the same exposures highlights the importance of choosing appropriate indicators, especially in consideration of the fact that the lipid metabolome is employed less often than the polar metabolome.

In addition to the traditional unheated approach polar metabolome samples were prepared following a modified heating protocol designed to denature enzymes and increase sample stability. Although both methods provided the same answer to the question, the data was not comparable between methods. When analyzed via PCA, the two methods produced two distinct clouds in ordination space. If data was to be used to create a reference condition, variation in methods would add unnecessary variability into the reference condition. It would follow therefore, that while a sample may be "out of reference" according to its analysis method, it may appear as "in reference" if several different methods were used to define the reference condition. Additionally, if a sample is analyzed using one method, it will not be comparable to reference data produced using the alternate method. This finding demonstrates a need for development and adoption of standard biomonitoring protocols so that data from multiple research groups can be compared.

Two environmental factors may have mitigated toxicity during this experiment beyond what was observed in the LC-50 tests from which I derived my test concentrations. First, it has been shown that naphthenic acids bind organic matter in a preferential manner as Janfada et al. (2006) found that naphthenic acids with carbon number ranging from 13-17 showed preferential sorption to sediment. My experiment utilized sediment with a TOC of 2%, suggesting that sediment binding could be influencing the observed toxicity to *Hexagenia spp*. by reducing the bioavailability of naphthenic acids. Second, the presence of abundant food has been shown to mitigate toxicity of several contaminants and therefore could have assisted the mayflies to tolerate the toxic effects of naphthenic acid exposure (Beketov Mikhail & Liess, 2009; Conley et al., 2011). Future assessments of naphthenic acid toxicity should consider cumulative stressors, such as

limited food availability and additional contaminants. Overall, this study provides a baseline for potential thresholds of safe release, however further research is needed to completely elucidate the environmental effects of naphthenic acid exposure on the environment.

3.6 References

Beketov Mikhail, A., & Liess, M. (2009). Acute contamination with esfenvalerate and food limitation: Chronic effects on the mayfly, *Cloeon dipterum*. *Environmental Toxicology and Chemistry, 24*(5), 1281-1286. doi:10.1897/04-256R1.1

Conley, J. M., Funk, D. H., Cariello, N. J., & Buchwalter, D. B. (2011). Food rationing affects dietary selenium bioaccumulation and life cycle performance in the mayfly *Centroptilum triangulifer*. *Ecotoxicology, 20*(8), 1840-1851. doi:10.1007/s10646-011-0722-1

Dokholyan, B., & Magomedov, A. (1983). Effect of sodium naphthenate on survival and some physiological-biochemical parameters of some fishes. *Journal of Ichthyology, 23*(6).

Frank, R. A., Kavanagh, R., Kent Burnison, B., Arsenault, G., Headley, J. V., Peru, K. M., . . . Solomon, K. R. (2008). Toxicity assessment of collected fractions from an extracted naphthenic acid mixture. *Chemosphere, 72*(9), 1309-1314. doi:http://dx.doi.org/10.1016/j.chemosphere.2008.04.078

Frank, R. A., Sanderson, H., Kavanagh, R., Burnison, B. K., Headley, J. V., & Solomon, K. R. (2009). Use of a (Quantitative) Structure–Activity Relationship [(Q)Sar] Model to Predict the Toxicity of Naphthenic Acids. *Journal of Toxicology and Environmental Health, Part A, 73*(4), 319-329. doi:10.1080/15287390903421235

Giesy, J. P., Anderson, J. C., & Wiseman, S. B. (2010). Alberta oil sands development. *Proceedings of the National Academy of Sciences of the United States of America, 107*(3), 951-952. doi:10.1073/pnas.0912880107

Hadwin, A. K. M., Del Rio, L. F., Pinto, L. J., Painter, M., Routledge, R., & Moore, M. M. (2006). Microbial communities in wetlands of the Athabasca oil sands: genetic and metabolic characterization. *FEMS Microbiology Ecology, 55*(1), 68-78. doi:10.1111/j.1574-6941.2005.00009.x

Headley, J. V., Peru, K. M., & Barrow, M. P. (2009). Mass spectrometric characterization of naphthenic acids in environmental samples: A review. *Mass Spectrometry Reviews, 28*(1), 121-134. doi:10.1002/mas.20185

Headley, J. V., Peru, K. M., Barrow, M. P., & Derrick, P. J. (2007). Characterization of Naphthenic Acids from Athabasca Oil Sands Using Electrospray Ionization: The Significant Influence of Solvents. *Analytical Chemistry, 79*(16), 6222-6229. doi:10.1021/ac070905w

Hsu, C. S., Dechert, G., Robbins, W., & Fukuda, E. (2000). Naphthenic acids in crude oils characterized by mass spectrometry. *Energy & Fuels, 14*(1), 217-223.

Humphries, M. (2008). *North American oil sands: history of development, prospects for the future*.

Janfada, A., Headley, J. V., Peru, K. M., & Barbour, S. (2006). A laboratory evaluation of the sorption of oil sands naphthenic acids on organic rich soils. *Journal of Environmental Science and Health Part A, 41*(6), 985-997.

Kinley, C. M., McQueen, A. D., & Rodgers Jr, J. H. (2016). Comparative responses of freshwater organisms to exposures of a commercial naphthenic acid. *Chemosphere, 153*, 170-178. doi:http://dx.doi.org/10.1016/j.chemosphere.2016.03.002

Leung, S. S., MacKinnon, M. D., & Smith, R. E. H. (2003). The ecological effects of naphthenic acids and salts on phytoplankton from the Athabasca oil sands region. *Aquatic Toxicology, 62*(1), 11-26. doi:https://doi.org/10.1016/S0166-445X(02)00057-7

Marentette, J. R., Frank, R. A., Bartlett, A. J., Gillis, P. L., Hewitt, L. M., Peru, K. M., . . . Parrott, J. L. (2015). Toxicity of naphthenic acid fraction components extracted from fresh and aged oil sands process-affected waters, and commercial naphthenic acid mixtures, to fathead minnow (*Pimephales promelas*) embryos. *Aquatic Toxicology, 164*, 108-117. doi:http://dx.doi.org/10.1016/j.aquatox.2015.04.024

Marentette, J. R., Sarty, K., Cowie, A. M., Frank, R. A., Hewitt, L. M., Parrott, J. L., & Martyniuk, C. J. (2017). Molecular responses of Walleye (*Sander vitreus*) embryos to naphthenic acid fraction components extracted from fresh oil sands process-affected water. *Aquatic Toxicology, 182*, 11-19. doi:http://dx.doi.org/10.1016/j.aquatox.2016.11.003

Melvin, S. D., Lanctôt, C. M., Craig, P. M., Moon, T. W., Peru, K. M., Headley, J. V., & Trudeau, V. L. (2013). Effects of naphthenic acid exposure on development and liver metabolic processes in anuran tadpoles. *Environmental Pollution, 177*, 22-27. doi:http://dx.doi.org/10.1016/j.envpol.2013.02.003

Schock, T. B., Strickland, S., Steele, E. J., & Bearden, D. W. (2016). Effects of heat-treatment on the stability and composition of metabolomic extracts from the earthworm *Eisenia fetida*. *Metabolomics, 12*, 47. doi:10.1007/s11306-016-0967-z

Swigert, J. P., Lee, C., Wong, D. C. L., White, R., Scarlett, A. G., West, C. E., & Rowland, S. J. (2015). Aquatic hazard assessment of a commercial sample of naphthenic acids. *Chemosphere, 124*, 1-9. doi:http://dx.doi.org/10.1016/j.chemosphere.2014.10.052

Taylor, N., Gavin, A., & Viant, M. (2018). Metabolomics discovers early-response metabolic biomarkers that can predict chronic reproductive fitness in individual *Daphnia magna*. *Metabolites, 8*(3). doi:10.3390/metabo8030042

Wang, J., Cao, X., Huang, Y., & Tang, X. (2015). Developmental toxicity and endocrine disruption of naphthenic acids on the early life stage of zebrafish (*Danio rerio*). *Journal of Applied Toxicology, 35*(12), 1493-1501. doi:10.1002/jat.3166

Xia, J. and Wishart, D.S. (2016) Using MetaboAnalyst 3.0 for comprehensive metabolomics data analysis current protocols in bioinformatics, 55:14.10.1-14.10.91.

Chapter 4

4 General Discussion

4.1 Summary

The overall goal of my thesis was to theoretically evaluate and empirically test the capabilities of the metabolome as a bioindicator for biomonitoring purposes. To accomplish this, my study had two components. First, an assessment of the suitability of the metabolome as an ideal bioindicator for environmental monitoring applications, and second, a test of the suitability of the metabolome as a bioindicator of naphthenic acid exposure for use in the Athabasca Oil Sands region of Western Canada.

4.1.1 Evaluation of the Metabolome as a Bioindicator

The first goal was accomplished through a thorough evaluation of available literature pertaining to current knowledge of metabolomics and its capabilities in an environmental context. Current biomonitoring goals present a need for a biomonitoring tool that is integrative of all levels of the biological hierarchy. While current methods have shown effectiveness at detecting change in aquatic environments, there are methodological limitations that prevent a holistic understanding of the ecosystem. Current methods generally fall under two approaches; top down methodologies which observe changes at a higher (e.g. community) level and aim to discern precursory changes at lower (e.g. biochemical) levels, and bottom up methodologies which observe changes at lower levels and aim to relate them to potential changes at higher levels (Munkittrick & McCarty, 1995). However, due to limitations of each approach, neither is capable of being fully integrative of the biological hierarchy, resulting in knowledge gaps and reduce capacity for management due to often poor integration of available tools (Allan & Castillo, 2007). To address this problem I evaluated the metabolome against nine criteria deemed to characterize an ideal bioindicator (Bonada et al., 2006).

I found that the metabolome, although not widely used as a bioindicator in biomonitoring, has significant potential for biomonitoring. The metabolome as a bioindicator is capable of

integrating biochemical responses with population level changes, overcoming a key limitation of many currently used methodologies. Moreover, I found that the metabolome has met or has the potential to meet all defined criteria for an ideal bioindicator.

The use of the metabolome is derived from sound theoretical concepts in ecology, as it is based in classic life history theory. Indeed, studies have demonstrated that as changes in resource allocation occur as a result of physiological compensatory processes, relative metabolite abundances shift and these shifts are captured in spectral differences in the measured metabolome. It has recently been shown that this linkage is not only theoretical, and that changes in the metabolome can be predictive of chronic fitness effects (Taylor et al., 2018). Although further research is needed to elucidate metabolomic pathways relevant to ecological change, the metabolome is often regarded as having *a priori* predictive capacity.

The metabolome is also capable of assessing ecological function within an ecosystem through knowledge of known ecological interactions. The metabolome has also shown capability in discriminating overall human impact and between different types of human impact (Skelton et al., 2014), although the reliability of such capabilities is yet to be determined due to limited use. Some stressors have shown linear metabolomic responses, which is often ideal for an indicator for biomonitoring purposes. Large scale applicability, while it has not been directly evaluated, is theoretically possible for the metabolome, as several evolutionary responses are conserved among different taxa.

Practically, the metabolome as a bioindicator represents little further expenditure with simple protocols that can be further simplified through the use of external analysis and interdisciplinary collaboration. Furthermore, the metabolome has the capacity to become an early warning and diagnostic indicator of ecosystem stress. However, the advancement of the capabilities of the metabolome is dependent on increased adoption and use. As the metabolome has theoretically and/or empirically shown to be capable of such benefits in addition to the established criteria for an ideal bioindicator, I concluded that the metabolome should be readily adopted into current biomonitoring programs.

4.1.2 The Metabolome as a Bioindicator of Naphthenic Acid Exposure

The second goal was accomplished through the use of a microcosm experiment where *Hexagenia spp.* were exposed to low concentrations of sodium naphthenate for a three week period. This study addresses the looming environmental problem caused by the large amassment of OSPW in tailings ponds and a current lack of direction regarding disposal or remediation. Knowledge is therefore needed into potential environmental effects of OSPW release to aquatic ecosystems. Naphthenic acids are considered the main toxic component of OSPW, and as such were assessed for exposure thresholds to provide direction for managers in the case of future release to the environment. As my thesis has demonstrated that environmental metabolomics has potential for biomonitoring, the metabolome was used as an indicator of exposure for mayflies to naphthenic acids in a controlled laboratory microcosm environment.

Following exposure, the cumulative metabolome of several groups of individuals were analyzed in order to determine metabolomic changes resultant from naphthenic acid exposure. The lipid metabolome showed a significant effect of exposure at the highest tested concentration (0.1 ug/L). However, the polar metabolome showed no indication of biochemical changes in exposed mayflies at the same tested concentrations. Moreover, no differences were seen in growth or survival at any concentration. Disruption of lipid bilayers has been proposed as a principal method of toxicity for naphthenic acid (Frank et al., 2009). The conclusion that the lipid metabolome responds prior to the polar metabolome therefore aligns with its proposed toxic mode of action.

Findings of my research demonstrate that the metabolome, when employed comprehensively, can function as a bioindicator of potentially adverse effects in the Athabasca Oil Sands region. More specifically my findings suggest that the lipid metabolome could be used as an early warning indicator of exposure to naphthenic acids. My work provides key insights into effective implementation of the metabolome for biomonitoring, both generally and specific to assessment of naphthenic acid exposure. Foremost, although the polar metabolome is the most commonly utilized portion of the metabolome, my result indicates that it is not always the first metabolome to respond. Specific to effective monitoring of naphthenic acids, my research indicates that more research is needed to elucidate concentration thresholds at which naphthenic acid exposure measurably impacts fitness. However, it has identified the potential for using the lipid metabolome as an early warning indicator of pending effects on organism fitness and

associated population effects. Such knowledge can be applied to managing expected adverse outcomes of naphthenic acids before they occur in the natural environment.

4.2 Applications and Recommendations

My findings show that when considering the metabolome for biomonitoring, it is important to consider the benefits of applying multiple complementary indicators. Although the polar metabolome is most commonly used, the lipid metabolome may in fact be more sensitive or faster responding in situations where the toxic mode of action of a contaminant preferentially effects lipids. When used together, the two metabolomes may represent a more holistic and effective view of ecosystem impacts. Additionally, if both metabolomes are not analyzed, it is important to consider the toxic mode of action of any stressor of interest. For example, if a stressor impacts mainly lipids, or lipids first, changes to organism fitness might not be observable in the polar metabolome (as demonstrated in the case of naphthenic acid toxicity to mayflies). Knowledge of traditionally utilized biomonitoring data alone therefore may not allow metabolomic data to be utilized to its maximum potential. While multiple indicators generally represent significant external expenditure, the combination of the polar and lipid metabolome is quite intrinsic as a single extraction will provide both metabolomes. As such, when considered in the context of practicality in biomonitoring, the combination of the polar and lipid metabolome represent multiple indicators that are more practical in comparison to the combination of several other indicators (e.g., algae and invertebrate communities).

I recommend that regional monitoring frameworks adopt metabolomics with input and recommendations from toxicologists. In order for the metabolome to be practically capable of its theoretical benefits, it is important to foster interdisciplinary collaboration. While ecologists have knowledge of sentinel species and their life history patterns, they may not be fluent in the organismal biochemistry required to elucidate the mechanisms responsible for observed declines in fitness. Conversely, while toxicologists excel at elucidating biochemical responses to contaminants, they may not have a full understanding of which species are relevant from an ecosystem monitoring and health perspective. Individually, the metabolome is capable of detecting change both in field and laboratory settings. However, if these disciplines were to see increased

integration, the diagnostic capability of the metabolome would be much easier to realize on a practical scale. If a difference is detected in routine biomonitoring assessments, the metabolomic pattern of change could be compared to distinct stressor fingerprints. This would allow causal predictions of toxicant stress to existing environmental (e.g., water chemistry) data. For this integration to occur, I suggest the development of a shared database between toxicologists and biomonitoring associations such that collected data can be effectively utilized by all stakeholders.

In addition to fostering interdisciplinary collaboration, I also recommend that it is important to foster collaboration among monitoring agencies. Although inherent variability in the metabolome has been recognized (see Chapter 2), there has been little recognition of the potential variability introduced by different analytical methods. Although a large interlaboratory comparison exercise concluded that data collaboration between laboratories is robust, the study utilized the same extraction and sample preparation procedure at each different location (Ward et al., 2010). A comparison of heated versus unheated metabolomic samples in Chapter 3 of my thesis clearly demonstrates that, although both methods will individually provide the same answer to the question, the data generated between the two methods is not comparable. In biomonitoring, it is often the case that a reference condition is established, and test sites are compared to the reference area. As both the heated and unheated methods produce distinct ellipses in ordination space, it is important to recognize that any reference condition established needs to do so with only one of the two methods and not both. It further follows that all test sites that are to be compared to the reference population must also follow the same analytical methods. Therefore, standardization of methods among biomonitoring agencies will be of utmost priority. To accomplish this, I recommend a standard set of analytical procedures for metabolomic biomonitoring be developed and published.

I recommend that further studies examining the metabolome for use in naphthenic acid monitoring take into account the potential for stressor specificity. Although any metabolomic response in an environmental context provides useful information, stressor specific responses would provide more detailed and therefore more useful information. Such information would enable diagnostic causal assessments that could in turn provide clear direction for protection and remediation. To accomplish this, I recommend that studies examine the impact of naphthenic acids in multiple stressor situations with stressors that would be concurrently present in environments where naphthenic acids are of concern.

I also recommend that further studies examining the metabolome for use in naphthenic acid monitoring take into account the affinity of naphthenic acid for sediment. As it is known that naphthenic acid binds sediment (Janfada et al., 2006), it will be important to understand the mechanics of the limits of sediment binding, and how toxicity is affected once those thresholds are surpassed. In biomonitoring for naphthenic acids specifically, this could direct concentration determination efforts to monitor either one or both of the sediment and/or water. Realistically, the metabolome would provide an indicator of exposure that is integrative of the exposure levels from both sediment and overlying water. However, it is important that these mechanics be established prior to determination of approved environmental concentrations.

4.3 References

Allan, J. D., & Castillo, M. M. (2007). *Stream ecology: structure and function of running waters*: Springer Science & Business Media.

Bonada, N., Prat, N., Resh, V. H., & Statzner, B. (2006). Developments in aquatic insect biomonitoring: a comparative analysis of recent approaches. *Annual Review of Entomology, 51*, 495-523.

Frank, R. A., Fischer, K., Kavanagh, R., Burnison, B. K., Arsenault, G., Headley, J. V., . . . Solomon, K. R. (2009). Effect of carboxylic acid content on the acute toxicity of oil sands Naphthenic acids. *Environmental Science & Technology, 43*(2), 266-271. doi:10.1021/es8021057

Janfada, A., Headley, J. V., Peru, K. M., & Barbour, S. (2006). A laboratory evaluation of the sorption of oil sands naphthenic acids on organic rich soils. *Journal of Environmental Science and Health Part A, 41*(6), 985-997.

Munkittrick, K. R., & McCarty, L. S. (1995). An integrated approach to aquatic ecosystem health: top-down, bottom-up or middle-out? *Journal of Aquatic Ecosystem Health, 4*(2), 77-90. doi:10.1007/BF00044791

Skelton, D. M., Ekman, D. R., Martinović-Weigelt, D., Ankley, G. T., Villeneuve, D. L., Teng, Q., & Collette, T. W. (2014). Metabolomics for in situ environmental monitoring of surface waters impacted by contaminants from both point and nonpoint sources. *Environmental Science & Technology, 48*(4), 2395-2403. doi:10.1021/es404021f

Taylor, N., Gavin, A., & Viant, M. (2018). Metabolomics discovers early-response metabolic biomarkers that can predict chronic reproductive fitness in individual *Daphnia magna*. *Metabolites, 8*(3). doi:10.3390/metabo8030042

Ward, J. L., Baker, J. M., Miller, S. J., Deborde, C., Maucourt, M., Biais, B., . . . Vervoort, J. (2010). An inter-laboratory comparison demonstrates that [1H]-NMR metabolite fingerprinting is a robust technique for collaborative plant metabolomic data collection. *Metabolomics, 6*(2), 263-273.

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McKenzie SM, Yates AG. (2018) 2017 Community Metabolism Study of the Athabasca River. Technical report prepared for Environment Canada, 11pp.

McKenzie SM, Yates AG. (2016) Community metabolism study of the Athabasca River. Technical report prepared for Environment Canada, 19pp.

Yates AG, McKenzie SM. (2016) Assessment of ecological function and structure of grassland streams in Canada. Technical report prepared for Parks Canada, 6 pp.

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